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Chronic stress and genetic influences on learning and memory in mice

by

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A dissertation submitted to the Graduate Faculty in Psychology (biopsychology subprogram) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

CHRONIC STRESS AND GENETIC INFLUENCES ON LEARNING AND MEMORY IN MICE

by

Meredith E. Kneavel

Advisor: Professor Victoria Luine

Investigation of environmental influences on phenotypic expression of diverse genotypes may provide insight into genetic vulnerability to disease and genomic factors involved in the stress response. The studies presented use null mutant (knockout) technology to begin to provide insight into these gene-environment interactions. Specifically, these studies present evidence that environmental stress has a significant effect on learning and memory, locomotion, and neurochemistry which is modulated by genetic background strain and the presence of specific proteins. Across the experiments, fourteen days of daily restraint stress generally enhanced both object recognition performance and radial arm maze and had minor if any effects on open field activity or neurotransmitter levels. Notably, significant differences in baseline behavioral performance and neurotransmitter levels between background strains was observed. In addition, this collection of studies has found that calbindin-D_{28k} is an important protein in mediating stress effects on learning and memory and that lack of calbindin-D_{28k} diminished the ability of the system to counteract harmful effects of stress. Alternately, results from these studies do not indicate that catechol-*O*-methyltransferase (COMT) plays a significant role in mediating stress effects on

learning or memory. What was revealed in the COMT studies, however, was the importance of background strain in expression of the null mutation. Results presented discuss the implications of these findings relevant to current knowledge of genetic differences, gene-environment interactions, and stress mechanisms.

Acknowledgements

This work is dedicated to all the people who were unfailingly supportive during this process, especially my advisor, Victoria Luine, my family- Mom, Dad, Chip, and Becky, and my best friend, David.

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FIGURE CAPTIONS

Chapter 1: Introduction

I. Stress and Cognitive Function

A. Stress

The study of stress includes several perspectives and definitions. These include stress as an environmental condition or challenge (McEwen & Mendelson, 1993; Dohrenwend & Dohrenwend, 1981a), appraisal of an environmental situation (Lazarus, 1976; Lazarus & Folkman, 1984), a response to that environmental situation (Selye, 1979; Cannon, 1939), or the relationship between environmental demands and ability to meet those demands (Taylor, Wood, & Lichtman, 1983; Taylor, 1983). Whatever the definition or perspective, stress threatens the homeostasis of an organism (Cannon, 1935). It is important to understand how these changes in homeostasis are affected and influenced by the central nervous system and specifically the brain. The central nervous system not only controls many aspects of the stress response and evaluation of a stressor, but also is the target of stress hormones. Further, understanding of genetic susceptibility to stress can provide understanding of the gene products involved in the stress response.

A recent focus of stress research has been its link to psychological and physical reactions. Prolonged or chronic stress can compromise the immune system response and is hypothesized to contribute to the onset of disease when preexisting genetic vulnerability exists (Kiecolt-Glaser & Glaser, 1991). Recent studies have shown that chronic stress impairs higher cognitive function (Nelson, 1999). Early research in the field of stress (Cannon, 1935) investigated the

physiological reactions that prepare an organism for the strenuous activity of either fighting or running away from a threatening stimulus. This fight-or-flight response involves activation of the sympathetic nervous system and endocrine responses and a return to homeostasis after the removal of the stressor (Cannon, 1935).

Selye further described responses to stress with what he termed the general adaptation syndrome (GAS). GAS is characterized by three stages (Selye, 1973a). During stage one, the organism experiences an alarm reaction which includes a shock phase involving initial and immediate reactions to a noxious agent and a countershock phase involving mobilization of defenses in which the adrenal cortex is activated and secretes corticoid hormones (Selye, 1973a). Stage two, the stage of resistance, involves the organism adapting to the stressor stimulus but becoming less able to cope with subsequent stimuli (Selye, 1973a). The final stage, the stage of exhaustion, follows a prolonged and severe adaptation of the stress response system. According to Selye (1979), stress induced arousal effects are adaptive if their impact is short term, however, as stress persists, chronic arousal can lead to exhaustion of the body's resources.

B. Physiology of Stress

Selye first proposed a role for the adrenal steroids in restoring homeostasis after stress episodes (Selye, 1979). He asserted that adaptation to stress requires the presence of adrenal steroids (glucocorticoids), but excess of these same steroids could lead to damage and disease commonly seen as a result of chronic stress (Selye, 1973b). This was later investigated to understand the exact role of

the adrenals in the stress response. Munck, Guyre, & Holbrook (1984) reported that the adrenal steroids counteracted the primary response of the body to stressors such as inflammation and increased heart rate, and, as postulated by Selye (1979), prolonged exposure to these adrenal steroids resulted in suppression of the protective responses.

Adrenal response to stress occurs through increased arousal of the hypothalamic-pituitary-adrenal (HPA) axis, Figure 1. The paraventricular nucleus (PVN) of the hypothalamus stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary, which triggers the release of glucocorticoids (GC) from the adrenal cortex (Herman & Cullinan, 1997; Jacobson & Sapolsky, 1991) resulting in increased plasma adrenal hormone levels (corticosterone in the rat and mouse, cortisol in humans). In addition to the physiological response to stress, the perception of an event as stressful increases activity in limbic and forebrain regions including the hippocampus, prefrontal cortex, amygdala, bed nucleus of the stria terminalis, preoptic area of the hypothalamus, and brainstem nuclei (Herman et al., 1997; Engstrom et al., 1990). These areas are implicated in the assessment of the environment and believed to modulate the HPA axis and thus, the stress response. Stress may also modulate the septo-hippocampal cholinergic system, which includes cholingeric pathways in the median septal area, dorsal hippocampus, lateral amygdala, and ventromedial hypothalamus and can mediate HPA functioning (Zerbib & Laborit, 1990).

There are many methods for studying stress. Some of the variation in stress research has to do with the diversity of definitions of stress mentioned

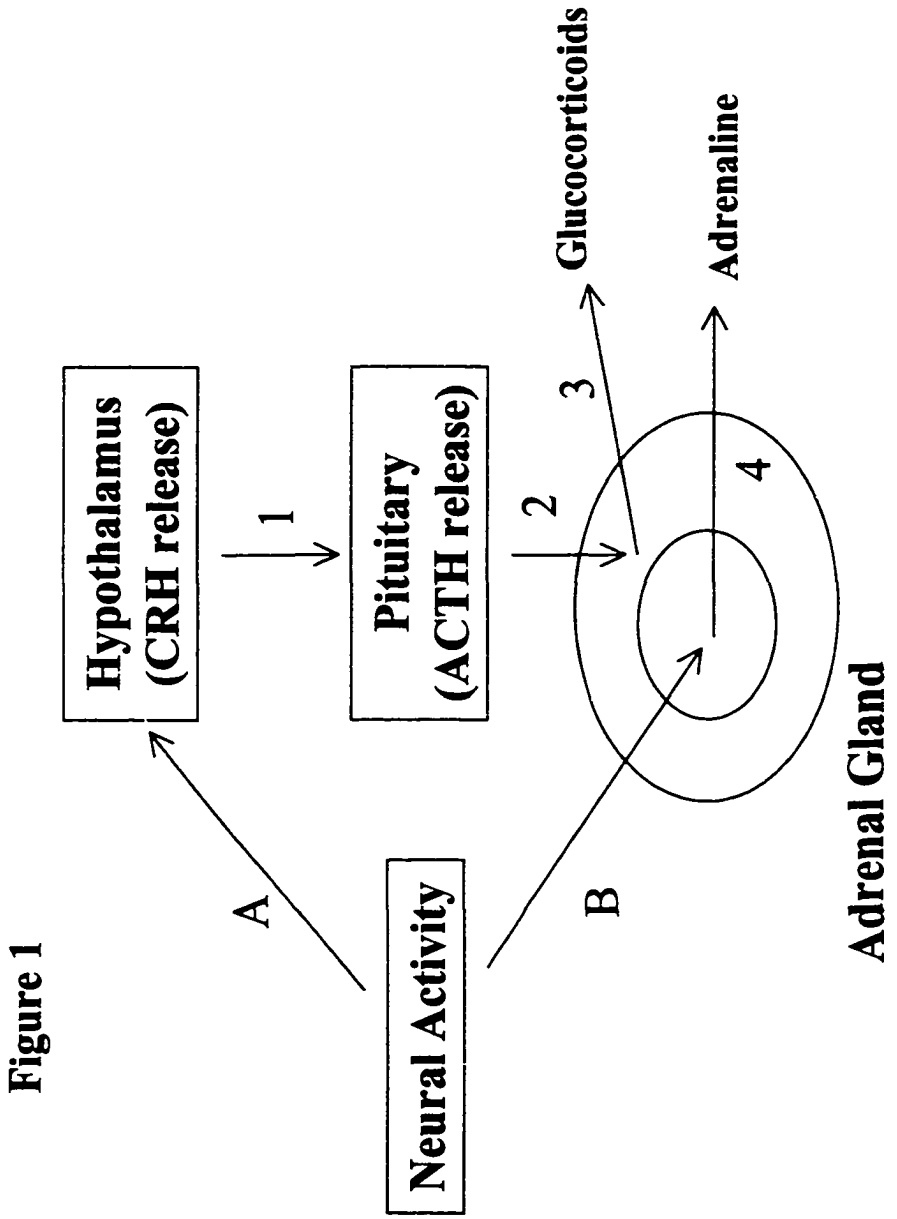


Figure 1

previously (e.g. see McEwen et al., 1993; Dohrenwend & Dohrenwend, 1974; Selye, 1973a; Cannon, 1935; Lazarus et al., 1984). In addition, the organism chosen to study the stress response influences the types of studies that are carried out. Further, stress can be classified as either acute or chronic, each having a unique but not unrelated set of responses (eg see (Shanks, Zalcman, Zacharko, & Anisman, 1991; Luine, Martinez, Villegas, Magarinos, & McEwen, 1996)). In animal models of stress, the physiological and behavioral effects have been tested using a variety of methods ranging from shock treatment, cold environments, prolonged and uncontrolled physical exertion, immobilization, restraint, noise, to flashing lights.

C. Brain Morphology and Stress

The effects of chronic stress on brain morphology and function as well as its effect on behavior have been investigated in animal models. Application of chronic stress (21 day restraint) or administration of exogenous corticosterone leads to alterations in the morphology of CA3 pyramidal neurons of the hippocampus including decreases in apical dendritic branching and dendrite length in male rats (Watanabe, Gould, & McEwen, 1992); (Magarinos & McEwen, 1995). The dendritic changes are mediated by glucocorticoids and can thus be prevented by inhibition of glucocorticoid secretion (Watanabe et al., 1992). In humans, evidence of hippocampal atrophy and loss of volume has been associated with sufferers of post-traumatic stress disorder who have increased levels of glucocorticoids (Sapolsky, 1996). One potential result of hippocampal neuronal destruction or alteration of this kind is a compromise in the ability of the

hippocampus to shut off pituitary-adrenal stress responses (Sapolsky, Krey, & McEwen, 1986), resulting in a chronic state of arousal.

Sex differences in morphological remodeling are currently being examined and suggest that female hormones modulate stress reactivity. Female rats undergoing chronic restraint stress show different patterns of hippocampal remodeling than stressed males (Galea et al., 1997). Stressed females have only very slight decreases in apical dendritic branching compared to stressed males, even though plasma corticosterone levels are higher in stressed females. Further, unlike stressed males, stressed females show significant decreases in the number of branching points in basal dendrites (Galea et al., 1997). Some of the sex differences in stress reactivity may be related to differences in hormonal states. Evidence from acute stress research indicates that elevated corticosterone levels in stressed female rats appear to be mediated by estrogen (Burgess & Handa, 1992; Handa, Burgess, Kerr, & O'Keefe, 1994). Specifically, ovariectomized females do not exhibit the high, prolonged corticosterone release seen in estrogen-treated or intact counterparts (Burgess et al., 1992). Further, naturally cycling females show the greatest level of corticosterone release in response to acute restraint during proestrus when estrogen levels are highest (Viau & Meaney, 1991). Thus, estrogen may be interacting with the HPA axis and increasing corticosterone levels; however, heightened corticosterone in females does not result in the same morphological changes seen in males, specifically, decreased apical dendritic branching and dendritic length.

D. Stress Dependent Neurochemical Changes: Monoamine Systems

Above cited morphological and other changes in response to stress influence gene activity and neurochemical signalling, which can influence the plasticity of the brain. Chronic stress and heightened levels of glucocorticoid release are known to alter neurochemistry (Sapolsky, 1996). Glucocorticoids have a modulatory effect on both the synthesis and release of dopamine, norepinephrine, and serotonin by regulating enzyme levels involved in synthesis or degredation and receptors of these neurotransmitters (McEwen et al., 1993). Stress or administration of corticosterone enhances (through activation of the adrenal gland) activity of the 5-HT precursor tryptophan hydroxylase in the midbrain and forebrain (Azmita & McEwen, 1974) and changes in 5-HT levels in response to stress may be modulated by the adrenal streroids (Azmita and McEwen, 1974). Ingestion of corticosterone, leading to higher plasma corticosterone levels, correlates with increased serotonin (5-HT) and serotonin metabolite, 5-hydroxyindole acetic acid (5-HIAA), levels in the dentate gyrus and decreased levels in the frontal cortex (Luine, Spencer, & McEwen, 1993). Decreased levels of 5-HT in the frontal cortex may reflect increased release and synthesis. In fact, some studies have found increased levels of serotonin and other neurotransmitters in the frontal cortex in response to stress (Adell, Garcia-Marquez, & Gelpi, 1989) which may be due to the inability of the system to increase synthesis in response to these increased neurotransmitter levels. In the hippocampus, alterations in 5-HT levels depend on the duration and severity of stress. Chronic, mild stress increases 5-HT activity in the hippocampus while

chronic, severe stress decreases it (Adell et al., 1989). Further, alterations in 5-HT levels may involve concurrent coping mechanisms. After activity-induced stress, development of gastric ulcers correlated with decreased 5-HT and 5HIAA, whereas failure to develop ulcers resulted in increased 5-HT and 5HIAA (Hellhammer, Hingtgen, Wade, Shea, & Aprison, 1983)). There is also evidence of sex differences in serotonergic changes in response to stress (Mendelson & McEwen, 1991); (Beck & Luine, 1998). Specifically, no change in 5-HT following chronic stress was observed in males, yet females had increased levels of 5-HT in the hippocampus following chronic restraint (Beck et al., 1998).

Dopamine (DA) related changes in response to stress also appear to be sexually dimorphic. In both the prefrontal cortex and the amygdala, males had an increase in DA levels in response to stress (Finlay, Zigmond, & Abercrombie, 1995) and a decrease in DA metabolism (Beck et al., 1998). Females, however, did not show any reduction in DA metabolism in these areas (Beck and Luine, 1998; Luine, 2000). Thus, it appears that neurochemical changes, DA in particular, due to stress are more apparent in the prefrontal cortical and amygdaloid regions of males. In females, neurochemical changes, particularly 5-HT, are more apparent in the hippocampal region.

Norepinephrine (NE) changes due to stress are also sexually dimorphic. Increases in norepinephrine in males following stress have been found in the amygdala and hippocampus (Beck & Luine, 1999). Further, NE turnover in the hippocampus increased with stress in males, whereas, no changes in NE were observed in stressed females in any of these brain regions (Beck et al., 1998).

Interestingly, ovariectomized females undergoing daily restraint stress howed increases in NE turnover (MHPG/NE ratio) in the CA3 region of the hippocampus.

While most of the neurochemical and morphological changes reported have been in rat models, some evidence is available from mouse studies. Although changes in neurotransmitters are less well documented in mice and no data exists for females, changes in both dopamine and norepinephrine in males have been observed. Increases in norepinephrine (NE) in mice following chronic stress found are similar to increases observed in rats (Shanks et al., 1991). Male mice undergoing chronic stress (2 hours per day/ 10 days) had increased norepinephrine (NE) metabolism in the locus coeruleus as well as the hypothalamus (Shanks, Griffiths, & Anisman, 1994). Studies have also found increased sensitivity of the dopaminergic system following chronic stress, though actual levels of this neurotransmitter have not been directly measured (Cabib, Kempf, Schleef, Oliverio, & Puglisi-Allegra, 1988; Puglisi-Allegra, Kempf, & Cabib, 1990).

Most of the research on effects of stress in mice has focused on changes in the cholinergic system. Changes in hippocampal cholinergic activity due to chronic stress are found in choline acetyltransferase activity, high-affinity [3H]choline uptake, and newly synthesized [3H]acetylcholine release (Finkelstein, Koffler, Rabey, & Gilad, 1985; Gilad, Rabey, & Shenkman, 1983). Pullia et al. (1996) found decreases in acetylcholine (Ach) levels following 10 and 14 days of restraint stress in mice. These levels returned to basal levels after 18

days of stress (Pullia et al., 1996). Further, muscarinic Ach receptors have been shown to up-regulate during chronic (5 to 10 days) stress and may down-regulate with longer (30 days) stress periods (Zerbib et al., 1990). Pharmacological evidence also indicates that chronic stress induces cholinergic supersensitivity to oxotremorine-induced hypothermia and oxotremorine-induced depression of locomotor activity (Zerbib et al., 1990); (Badiani, Castellano, & Oliverio, 1991)). These cholingergic changes may have profound impacts on learning and memory as discussed below.

E. Stress Dependent Neurochemical Changes: Amino Acids

Stress induced structural changes in the CA3 neurons of the hippocampus may be mediated by glutamate and GABA, amino acids involved in relaying information from the mossy fibers of the dentate gyrus to the CA1 neurons of the hippocampus. Chronic restraint stress increased hippocampal GABA and glutamate levels in males but not females (Beck et al., 1998). In addition, stressed males had increased release of glutamate levels in the frontal cortex, a brain region which can mediate hippocampal function (Moghaddam, 1993). In addition, pharmacological studies have found evidence of the role of excitatory amino acids in the stress response. Phenytion, which interferes with glutamate activity, blocks the atrophy of the CA3 dendrites as well as attenuates radial arm maze impairments associated with stress (Luine & Rodriguez, 1994b).

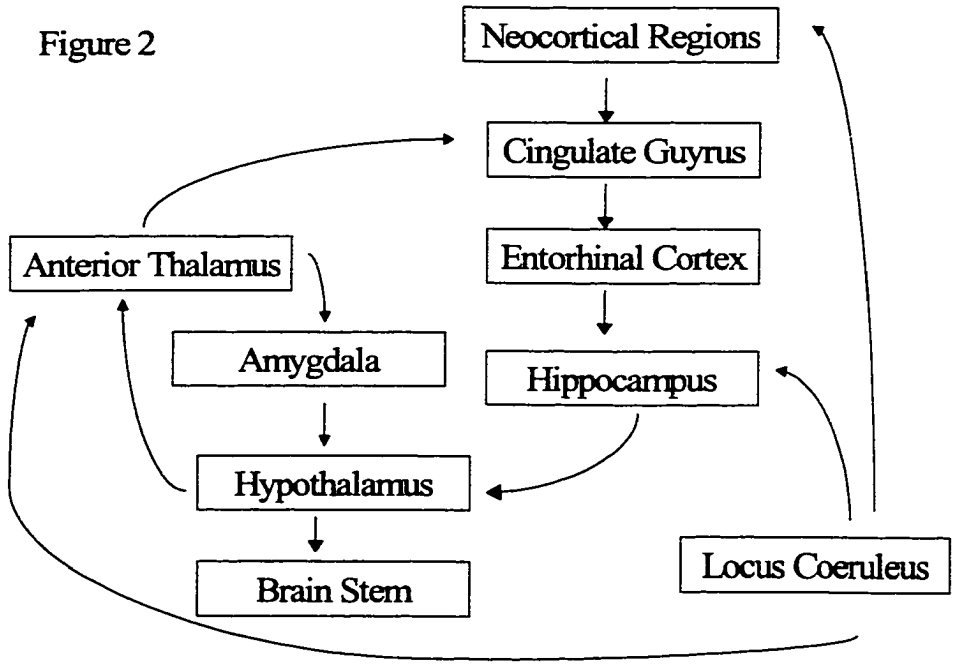
F. Stress Dependent Modulation of Learning and memory

As discussed above, stress results in several neurochemical alterations. These changes have implications for learning and memory since many of the

changes occur in brain regions involved in cognitive function, Figure 2. As proposed by Luine (Luine, Villegas, Martinez, & McEwen, 1994a; Luine, 1997), degeneration of the hippocampal neurons due to stress is associated with learning and memory deficits. Long-term potentiation (LTP) and primed burst potentiation (PB), neuronal substrates of learning and memory formation are also affected by stress. LTP is a long lasting increase in the excitablity of postsynaptic neurons and often an increase of dendritic growth due to repeated electrical stimulation. PB is a low-threshold form of LTP that also contributes to learning and memory formation. Stress and associated high corticosterone levels have been shown to block both LTP (Foy, Stanton, Levine, & Thompson, 1987) and PB (Engstrom et al., 1990; Diamond, Fleshner, & Rose, 1994). In addition, deficits in spatial memory following chronic stress include impairment on the spatial Y-maze (Conrad, Galea, Kuroda, & McEwen, 1996), Morris water maze (Bodnoff et al., 1995), radial arm maze (Luine et al., 1994a) and Barnes maze (Maclay, Freeman, & Zadina, 1998). Further, non-spatial memory performance has also been found to be impaired following 21 days of chronic restraint in males (Beck et al., 1999). The stress effects are reversible after removal of the stressor and are dependent on the length of the stressor. In fact, a shorter 14-day stress regimen enhanced radial arm maze performance in male rats (Luine et al., 1996).

As expected, based on sex differences in morphological changes and neurotransmitter changes in response to stress, stress effects on learning and memory performance are different in males and females. Specifically, females, unlike males, are enhanced on the radial arm maze following 21 days of stress

Figure 2



(Bowman, Zrull, & Luine, 2001). Further, non-spatial memory impairments seen in male rats on the object recognition task were not found in females (Beck et al., 1998). The spatial object placement task reflects the sex differences observed on the radial arm maze as well (Beck and Luine, submitted). Further, females exhibit less impairment following stress (restraint or shock) in acquiring avoidance learning (Kirk & Blampied, 1985; Steenbergen, Heinsbroek, Van Haaren, & Van de Poll, 1989; Steenbergen, Heinsbroek, Van Hest, & Van de Poll, 1990) or escape conditioning (Davis, Porter, Burton, & Levine, 1976). Other evidence of the differential effects of stress on males versus females comes from acute stress studies in which females have been found to be impaired on a variety of memory tasks and males are not. For instance, in trace eye blink conditioning, acutely stressed females have impaired performance while males show enhanced performance (Wood & Shors, 1998), however, this effect is not seen in ovariectomized-stressed females (Shors, Lewczyk, Pacynski, Matthew, & Pickett, 1998). Taken together, these results suggest that stress differentially affects the sexes or that some hormone or neurochemical factors may be different in males and females.

II. Use of genomically altered mice to understand stress effects on cognition

Based on these findings, investigation of stress effects on cognition should examine areas related to both stress and learning and memory. Specifically, areas involved in learning and memory include the prefrontal cortex, which is involved in attentional aspects related to learning. Ablation of the frontal cortex can disrupt non-spatial memory (Ennaceur, Neave, Aggleton, 1996). As shown in the

schematic of the learning and memory circuit (Figure 2), the prefrontal cortex, part of the neocortex, projects to the cingulated gyrus which in turn projects to the entorhinal cortex. Recently the entorhinal cortex has been shown, through ablation studies to be necessary for non-spatial object recognition performance (Ennaceur, Neave, Aggleton, 1997). The entorhinal cortex projects to the hippocampus, an area long known to be involved in spatial memory (Olton & Papas, 1979). The hypothalamus then projects to areas of the brain stem and the anterior thalamus. The anterior thalamus then projects back into the learning areas through the cingulate gyrus, completing the circuit. In addition, the amygdala, which lies just rostral to the hippocampus, is part of the limbic system and involved in motivation and emotion. The amygdala receives sensory information from the sensory cortex and thalamus and projects to the hypothalamus. In addition to the amygdala, the locus coereleus is involved in arousal and projects to several areas in the learning circuit.

In addition to hormonal effects on stress, genetic differences may also mediate vulnerability to stress effects on biochemical, morphological, and behavioral measures. Several studies have reported significant strain differences in mice in reaction to stress (Table 1). These strain differences most likely reflect underlying genomic variation. Strain differences have been found using a variety of stressors including restraint and footshock with a variety of durations ranging from acute to chronic in C57BL/6, DBA, CD1, and BALB mice (Table 1). However, few studies have characterized stress reactivity of the 129 strain, which is frequently used as a background strain in the creation of transgenic and

knockout mice (Crawley, 2000). Thus, it is important to characterize and compare the behavior and biochemistry in the two strains of mice commonly used in the creation of transgenic and knockout models. Characterization of these strains will provide baseline information about these mice before the genetic manipulation. Furthermore, if these strains or mutants created on these strains will be used to investigate possible mediators of stress effects, then stress effects in each strain should be identified.

A. Creation of transgenic and null mutant mice

One of the limitations of research with transgenic and knockout mice is that they are usually a mixture of two strains, the 129sv and C57Bl strains. Therefore interpretation of behavioral and biological functioning may be clouded by the possible influence of background strain on which the mutant line is maintained and created (Crawley, 1999). The choice of the background and donor strains usually relates to features of the donor cells, maternal behavior of the mice, probability of successfully carring the embryo to full term, and several other factors discussed below.

Transgenic mice are created by insertion of a gene into the genome. The gene may be a new gene that has been added such as the human gene for Huntington's disease (Carter et al., 1999) or a copy of an existing gene such as corticotropin releasing factor (Stenzel-Poore, Heinrichs, Rivest, Koob, & Vale, 1994). Transgenic models are created to investigate excessive expression of a hormone or particular protein. Creation of the transgenic mouse involves microinjection of the new or extra gene in the DNA construct into the pronucleus

of the fertilized mouse oocyte. The microinjected oocytes are then implanted into a pseudopregnant female. The new or extra gene becomes incorporated into the genome through random homologous recombination (Crawley, 2000). If this recombination occurs before the first cell division of the embryo, all cells in the developing mouse incorporate the new or extra gene. If the recombination occurs later in the development of the embryo, a genetic mosaic, in which some cells contain the new or extra gene and others do not, is created. For behavioral, neurochemical, and neuroanatomical evaluation, it is important to note where and on which chromosome the transgene has inserted itself in the DNA (Crawley, 2000).

Knockout or null-mutant mice are created through targeted gene mutation techniques, which are designed to delete or inactivate a specific gene. The mutation is a specific deletion of a portion of DNA, which is critical for expression of a particular gene product. Often the mutation is a shift in the reading frame for the DNA, rendering incorrect reading of the triplet base pair codes for the amino acids that make up the gene product. The first step involves creation of a targeted gene construct, which contains the targeted gene that is being 'knockout out' or turned off. In addition, an antibiotic resistance gene, usually neomycin resistance gene (*Neo*') is inserted into a critical region of the targeted gene. The neomycin resistance gene serves as a tag to identify gene constructs that contain the mutated or knocked out gene. Embryonic stem (ES) cells harvested from the inner cell mass of blastocysts are then cultured and electroporated with the targeted gene construct containing the antibiotic resistance

Table 1: Stress Research in Mice

Authors	Stress Method	Stress Duration A= Acute C= Chronic	Strain(s)	Measure	Findings
(Shanks et al., 1994)	Footshock	C: 15 days	C57 & BALB	NE; 5HT	BALB more variation in NE & 5HT; NE, MHPG ↑hypothal; ↓LC & Fctx
(Cabib & Puglisi-Allegra, 1991)	Restraint	C: 10 days	DBA; C57	climbing, apomorphine (APO), DA, DOPAC, HVA, 3MT	DBA: stress + 0.25 mg/kg APO → ↑climbing C57: stress + 0.25mg/kg APO → ↓climbing DBA & C57: stress to APO → ↓DOPAC, HVA, 3MT in CP & NAS
(Puglisi-Allegra et al., 1990)	Restraint	C: 2 hrs/ day/ 10 days	C57 & DBA	DA related behavior (apomorphine response) locomotion	C57 hypersensitive DA autoreceptors, increase in D1/D2 receptor ratio, decrease spontaneous climbing behavior (novel environment)
(Shanks et al., 1991)	Footshock (inescapable)	A: 1 session	AJ; BALB; C3H; C57; DBA; CD1	NE; DA; 5HT and metabolites	↓NE & ↑MHPG in hypothal. of all strains. Other brain regions- many strain differences
(Cabib et al., 1988)	Restraint	A: 30 minutes, 120 min	C57; DBA; B6D2F1	DA and metabolites	↑DOPAC/DA &HVA/DA ratio, ↓3MT/DA ratio after 120 in CP, after 30 min in NAS. No change Fctx in C57; ↓HVA/DA rat. in Fctx of DBA; DBA dominant inheritance
(Hirano, Nagai, Bando, & Nakagawa, 1991)	Restraint	C: 2 hrs/ day/ 8days	Jla:ddy	[^{3}H] DA in adrenal chromaffin cells	stress→↑DA uptake in adrenaline storing chromaffin cells
(Pullia et al., 1996)	Restraint	C: 2 hours/ day : 10 days, 14 days, 18 days	DBA	oxotremorine effects of locomotion; Ach levels	10 & 14 days stress + oxotremorine→↓oxotremorine sensitization & ↓Ach in Prefrontal cortex
	Footshock (inescapable and escapable)	A: 1 session C: 15 days	CD1	Immobiliity in forced swim task	initial increase in swimming then decrease. No effect of controllability of stressor
(Cabib & Castellano, 1997)	Restraint Food restriction	A (R): 15, 30, 60 min	DBA	Inhibitory avoidance memory (step-	DA receptor agonist magnified stress impairment (↓) in step-through latency

		C (FR): 13 days		through latency)	
(Talan & Engel, 1989)	Cold exposure, partial restrain	C: 3 hr/ 2 weeks/ 4 weeks	C57	Electrical self stimulation of hypothalamus (ISS)	cold acclimation prevented by ISS of reward area
(Calvo-Torrent, Brain, & Martinez, 1999)	Predatory exposure (rats) food and water deprive	A: 3 hours C: 21 days	CD1	Sucrose intake; anxiety (+-maze)	acute stress→↑fecal boli on +-maze chronic stress→↓sucrose intake, ↑anxiety
(Zacharko, Gilmore, MacNeil, Kasian, & Anisman, 1990)	Footshock	A: 1 session	BALB; C57; DBA	intercranial self-stimulation (ISS)	C57 no change; BALB immediate ↓ in ISS; DBA immediate, 24 hr, & 168 hr after stress ↓ ISS. Strain diff.
(Kaneto, 1997)	Footshock (FS) Psycholo. (PSY)* Swim stress (SW)	A: 5 min	ddy	passive avoidance, scopolamine effects	Pretrain + PSY → facilitation of step-through; pretrain + SW → impairment; FS or control + scopolamine → impaired * PSY = hearing and seeing footshock
	Footshock	A: 1 session C: 5 days	CD1	immune response	Footshock → decreased immune response
(Konecka, 1992)	Restraint	A: 15, 30, or 60 min.	CFW	opiod system; Ach	30 min →↑adrenal Ach; 30 + naloxone → no ↑ Ach
(Badiani et al., 1991)	Restraint	A: 2 hours C: 2 hrs/ day/ 14 days	DBA; C57	locomotion, oxotremorine sensitization	Acute stress + oxotremorine →no change in locomotion chronic stress + oxo →sensitization in DBA but not C57
(Irwin & Livnat, 1987)	Footshock Restraint Tailshock Cold water	A C	DBA; C57; CD1	Immune response Review article	Acute FS→↓natural killer cells in CD1 & C57 no DBA chronic stress →no change
(Shanks, Griffiths, Zalcman, Zacharko, & Anisman, 1990)	Footshock	A: 1 session C: 14 days	AJ; BALB; C57; C3H; DBA; CD1	plasma cort levels	Acute and chronic stress →↑plasma cort levels return to baseline levels was strain dependent

(Zerbib et al., 1990)	Restraint	A (2x2 hours/1 day) C: 10 days or 30 days, 5x/wk	OF1	passive avoidance; scopolamine; oxotremorine	stress + scopolamine → ↑amnesia in passive avoidance, stress + oxotremorine → ↑sensitivity to hypothermia
(Willner, Moreau, Nielsen, Papp, & Sluzewska, 1996)	Prevent wire gnawing	C (10 days)	Zur:ICR	stereotypy; cort levels	↑cort, no change in stereotypy
(Wurbel & Stauffacher, 1996)	Chronic mild stress*	C	CD1 & rats	body weight; hedonic response	stress→ ↓sucrose consumption, weight loss of 6-10%
(Pardon, Perez-Diaz, Joubert, & Cohen-Salmon, 2000)	Chronic mild stress*	C: 8 weeks *see below	B6D2F1	T-maze	stress →higher frequency of alteration responses
(Badiani, Cabib, & Puglisi-Allegra, 1992)	Restraint	C (10 days)	C57; DBA; B6D2F1	locomotion; amphetamine response, DA	sensitization to stimulatory effect of amphetamine in locomotion in DBA but not C57, DBA dominant mode of inheritance, no change in DA, HVA, 3MT
(Maestripieri, Badiani, & Puglisi-Allegra, 1991)	Restraint Novel Environment	C: 2hrs/ day/ 10 days	Pregnant swiss outbred	anxiety; aggression	stress →increased maternal care, ↑anxiety, ↓maternal aggression

- Chronic ultramild stress: application of a variety of mild stressors including: repeated periods of cage tile (30°); confinement to small cages (11 x 8 x 8 cm); 2-2 hour periods of paired housing; 1 overnight period of difficult access to food (without a reduction in the daily food ration); 1 period of continuous overnight illumniation; or 1 overnight period in a soiled cage (50 mL water in 1000 mL of sawdust bedding). Stressors were scheduled over a 1-week period and repeated throught the 8-week experiment. Except for the weekend, animals were subjected to a single 1-hour period of morning stress, a single 2-hour period of afternoon stress; and an overnight stress with a minimal interval of 2 hours between each stress-inducing period

List of current studies involving chronic or restraint stress and strain differences. It is important to note that there are no studies reporting direct comparisons of the effects of chronic restraint stress in C57BL/6 and 129Sve strains.

marker and the knockout. Electroporation involves delivering an electrical current through the ES cells which opens the 'pores' or channels in the ES cell membrane nucleus thus permitting entry of the DNA containing the new targeted gene construct. When the current is turned off, the membrane closes and the ES cell is returned to its normal state, apparently undamaged. Through random homologous recombination, some of the ES cells incorporate the targeted gene construct into the genome. ES cells are often harvested from 129 strains because this strain readily yields robust embryonic stem cell lines that maintain pluripotency after extended in vitro culture and electroporation (Crawley, 2000).

The ES cells are grown in a tissue culture containing a solution of antibiotic, most often neomycin. Therefore, those ES cells which have incorporated the antibiotic resistant (i.e. neomycin) and, thus, the targeted gene construct survive. These cells, which are known to contain the targeted mutation, are then cultured and implanted into a blastocyte harvested from other mice. Often the blastulas are collected from superovulated female mice of the C57BL/6 inbred strain because this strain is easily attainable and readily yields blastocytes. The injected blastulas are then implanted into other pseudopregnant female recipients also usually of the C57BL/6 or CD-1 strains because these strains maintain pregnancies well and provide good parental care. The resultant mouse is often a chimera, which contains a mixture of cells from the 129 and C57BL/6 or CD-1 strains, as well as the targeted mutation. The challenge to researchers is characterizing the effect of the mutation on this mixed background. In order to characterize the influence of a genetic manipulation, it is important to first

establish familiarity with baseline behaviors in the background strains used to create the mutant.

B. Null Mutant and Transgenic Models as Tools: Usefulness in studying the stress response

Null mutant and transgenic mice may be useful in studying the mechanisms of stress vulnerability. Specifically, null mutant and transgenic techniques may provide a window for understanding environmental influences on genetic mechanisms. Recently, a few studies have begun to investigate the effect of stress in genetically altered models. For example, this approach has been used to investigate the effects of oxidative stress in subjects with mutations of the antioxidant glutathione peroxidase (Jiang, Akopian, Ho, Walsh, & Andersen, 2000), inducible nitric oxide synthetase (Calingasan, Huang, Chun, Fabian, & Gibson, 2000; Calingasan, Chun, Park, Uchida, & Gibson, 1999), and superoxide dismutase (SOD-1) (Chan et al., 1995). Results have provided insights into the mechanisms of oxidative stress and found for instance that a 3-fold increase in SOD-1 provides increased protection against cerebral ischemia, decreases in acute injuries such as brain edema and blood-brain barrier permeability, and decreased neurological deficits following traumatic brain injury (Chan et al., 1995). In addition, increased resistance to epileptic seizures was found in glutamate peroxidase null mutants suggesting that chronic use of antioxidants may be deterimental rather than helpful to those at risk for seizures and brain injury (Jiang et al., 2000). Work with the inducible nitric oxide (iNOS) knockout has provided evidence that cell specific oxidative stress is associated with neurodegeneration

during thiamine deficiency, which is not mediated by iNOS (Calingasan et al., 1999).

A limited number of studies have examined the effects of chronic stress in null mutant or transgenic models. However, orphanin FQ/nociception (OFQ-N) null mutants have increased sensitivity to stressful stimuli and lack habituation to chronic stress (Reinscheid, Nothacker, & Civelli, 2000). In addition, the corticotropin releasing hormone (CRH) null mutant was used to investigate the role of this protein in food intake alterations induced by chronic stress (Weninger, Muglia, Jacobson, & Majzoub, 1999). Contrary to previous pharmacological studies, which blocked CRH, deletion of CRH from the genome did not reduce eating in response to chronic stress (Weninger et al., 1999). These findings also illustrate the important point that pharmacological findings do not necessarily coincide with or support null mutant results. Together these methods can provide a clearer picture of the exact mechanism of action, any gene- or dose-response effects, and potential developmental influences of a particular gene product. Further studies with null mutants found interleukin-6 (IL-6) to be an important protein in increasing corticosterone levels, increasing arginine vasopressin, but not in changing adrenocorticotrophic hormone (ACTH) levels in response to stress (Raber, O'Shea, Bloom, & Campbell, 1997). These results suggest that activation at the level of the adrenal gland may be directly related to argine vasopressin release or sensitization to ACTH (Raber et al., 1997). Finally, work with Apo-E null mutants has found that exposure to chronic stress, like aging,

increased autoimmune responses associated with neurodegeneration (Zhou, Elkins, Howell, Ryan, & Harris, 1998).

Work with null mutants and transgenics as a tool for understanding the mechanisms of the stress response has just recently begun. A variety of null mutant and transgenic models have been created to mimic diseases and increase understanding of these disorders. It has been hypothesized that stress, particularly chronic stress, may exacerbate the symptoms or onset of several of these diseases (Dumery et al., 2001). Transgenic and null mutant models provide a mechanism with which to study the potential of genetic vulnerability to stress. Further, even in models where stress may not be suspected as related to disease onset, environmental influences on null mutant and transgenic models should not be ignored.

In support of environmental influences on gene function, many studies with humans have correlated psychological disease onset with environmental stressors or dysfunctional familial relations. The vulnerability hypothesis states that chance exposures to stressors can trigger illness onset in already vulnerable people (Dohrenwend & Dohrenwend, 1981b). More specifically, the diathesis-stress model of schizophrenia indicates that stress will exacerbate or precipitate predisposed schizophrenic symptoms. Recent studies have found a link between stressful life events and onset of schizophrenia (Norman & Malla, 1993a; Norman & Malla, 1993b). A longitudinal study of schizophrenic patients reported exacerbation of existing symptoms with increased incidence of life event stress (Ventura, Nuechterlein, Hardesty, & Gitlin, 1992). Thus, the role of the

environment in contributing to diseases and disorders has long been an area of interest. One of the difficulties with these studies however, is establishing the relative time of stress in relation to onset of disease. For instance, Brown and Harris found that only three weeks preceeding psychiatric illness onset patients were significantly different from control in the number of life events experienced (Brown, Harris, & Peto, 1973). Yet, Ursano et al. (1981) found that Vietnam prisoners of war showed signficant differences in psychiatric disease manifestation depending on severity of stress during imprisonment five years after release, though no signficant differences were found at the time of release (Ursano, Boydstun, & Wheatley, 1981).

Investigation of the interaction between heredity (genes) and the environment in animal models was previously not possible. Specifically manipulating genes and their expression could only be investigated as a result of random mutations and subsequent backcross breedings. Transgenic and knockout mice have now made it possible to study the interaction of genes and the environment by manipulating both. Consistent with Mendelian genetics, the knockout mice can be heterozygous, (contain one copy of the gene and one blank); homozygous (not contain any copies of the gene); or wild type (contain two copies of the gene), as in a normal mouse. The wild type mouse comes from the same mother-father pair as the heterozygous and homozygous and thus is over 95% genetically similar except for the target gene after at least six backcrosses.

C. Null Mutant and Transgenic Models as Tools: Usefulness in studying learning and memory processes

Just as null mutant and transgenic mice are useful in studying the mechanisms of stress, they are also useful in studying the mechanisms of learning and memory. According to Mayford et al. (1995), knockout and transgenic techniques to neurobiology will provide a bridge between genes and cognition. Specifically, these genetically altered mice make it possible to explore molecular mechanisms underlying implicit and explicit forms of learning, short-term and long-term memory, and emotional behaviors. The analysis of these mutant mice has begun to link specific behavioral deficits to defined changes in synaptic physiology. These null mutants or knockouts allow for investigation of learning and memory processes that compliments current pharmacological and ablation studies.

Many studies recently have assessed the effects of specific genetic knockouts on learning and memory. For instance, impairments in memory have been found in corticotropin releasing factor receptor knockouts on the Y-maze(Contarino et al., 1999), dopamine D1 receptor knockouts on the Morris Water maze (El Ghundi et al., 1999), and receptor-associated protein (RAP) knockouts on the Morris water maze (Van Uden, Veinbergs, Mallory, Orlando, & Masliah, 1999). Other studies have shown that deletion of particular genes does not affect learning. For instance, mice lacking the cell adhesion molecule Thy-1 showed normal learning on spatial memory tasks and in the T-maze using scented food as cues (Mayeux-Portas, File, Stewart, & Morris, 2000). Alternatively, some

null mutants exhibit superior performance compared to wild-type controls. For instance, nociceptin-knockout mice show enhanced learning ability in the Morris water maze task, show enhanced latent learning in the water finding task, have better memory performance in a passive avoidance task, and show larger long-term potentiation in the hippocampal CA1 region compared to wild-type controls (Nabeshima, Noda, & Mamiya, 1999). Several studies have also evaluated the role of specific genes in memory recovery from traumatic brain injury, e.g. see (Whalen et al., 1999a; Whalen et al., 1999b). Knockouts have also been created to mimic human conditions that involve memory impairments: phenylalanine hydroxylase (Pah)-deficient "PKU mice" have a mutation in the Pah gene which is known to cause phenylketonuria (PKU) in humans. Knockout of the Pah gene in mice results in cognitive impairments that mimic those seen in humans with PKU (Zagreda, Goodman, Druin, McDonald, & Diamond, 1999).

III. Thesis Aims

This thesis will address some of the challenges of working with and characterizing null mutants in order to gain understanding of the stress response. Initially, establishment of behavioral paradigms (open field, object recognition, radial arm maze) and neurochemical analysis of several brain regions in wild-type mice will be carried out. Next, investigation of specific mechanisms hypothesized to be involved in the stress response will be attained through work with the effect of stress in null mutants.

Each study will be designed to answer two main questions.

Study 1:

Question 1: Do differences exist between C57BL/6 and 129SvE strains in behavioral and neurochemical measures?

Aim 1: Obtain baseline comparison of 2 strains on paradigms of interest: spatial and non-spatial learning and memory, open field activity, and neurochemical profile

Question 2: Do differences exist between these strains in the stress response?

Aim 2: Compare stress response in C57BL and 129Sve strains on these measures

Study 2:

Question 1: Does calbindin-D28K null mutation affect learning and memory performance, activity levels, or neurochemical profiles?

Aim 1: To characterize the effect of the null mutation on learning and memory, locomotor activity, and neurochemical levels in several brain regions

Question 2: Does calbindin-D28K null mutation increase stress vulnerability?

Aim 2: To characterize the role of calbindin in the stress response

Study 3:

Question 1: Does catechol-*o*-methyltransferase (COMT) deletion in females affect learning and memory performance, activity levels, or neurochemical profiles?

Aim 1: To characterize behavioral and neurochemical profiles of COMT null mutants

Question 2: Does background strain influence expression of null mutation?

Aim 2: To characterize COMT null mutation on both the C57BL and 129Sve background.

CHAPTER 2:
Comparison of stress effects in C57BL/6 and 129/SvEvTac mice strains: Physiological and cognitive profiles.

INTRODUCTION

Differences in learning and memory have recently been reported between various mice strains (Nguyen, Abel, Kandel, & Bourtchouladze, 2000; Owen, Logue, Rasmussen, & Wehner, 1997). These studies suggest significant strain differences in a variety of memory tasks between C57BL/6J and 129/SvEvTac strains, two strains commonly used in the creation of null mutant and transgenic mouse models (Owen et al., 1997; Wehner & Silva, 1996). Interestingly, the 129/SvEvTac and other 129 strains have an incomplete or missing corpus collosum, among other neuroanatomical differences, which may partially explain the observed behavioral differences (Balogh, McDowell, Stavnezer, & Denenberg, 1999; Wehner et al., 1996). Genetic analysis using null mutants derived from these inbred strains is routinely being conducted to identify determinants of normal behavior and pathological states (Crawley et al., 1997). Therefore, it is important to characterize and evaluate cognitive performance in the strains used to create null mutants.

In addition to possible strain differences in behavior and cognitive function, there may also be differences in reactivity to environmental stimuli. Recent reports have found significant differences in several behaviors, including

learning and memory, across several laboratories that may be attributable to minor environmental changes (Crawley et al., 1997; Owen et al., 1997). Chronic restraint stress has been shown to affect behaviors ranging from passive avoidance (Zerbib et al., 1990; Cabib et al., 1997), to spontaneous nest leaving (Stone, Zhang, & Quartermain, 1997), locomotion (Puglisi-Allegra et al., 1990); (Badiani et al., 1991; Badiani et al., 1992), and anxiety and aggression in mice (Maestripieri et al., 1991). Some of these chronic stress effects are compared across strains; however, these studies are generally limited to strains other than 129 (Crabbe, Wahlsten, & Dudek, 1999; Owen et al., 1997). Little information exists on the effect of chronic stress on cognitive function in mice or whether stress affects strains differently (Badiani et al., 1991; Badiani et al., 1992); yet, previous studies have found consistent effects of chronic restraint stress in rats. Spatial (Luine et al., 1996) and non-spatial (Beck et al., 1998) memory performance is enhanced by short periods of stress and impaired by long periods.

The stress response may be mediated by or affect neurotransmitters in the brain, particularly in areas on the hypothalamic-pituitary-adrenal (HPA) axis. Specific effects on neurotransmitters are well documented for acute stress and include significant strain differences in dopamine (DA), the dopamine metabolites (DOPAC and HVA), norepinephrine (NE), NE metabolite (MHPG), serotonin (5HT), and 5HT metabolite (5HIAA) levels in several brain regions including locus coeruleus, ventral tegmental area, mesocortex, prefrontal cortex, and hypothalamus (Shanks et al., 1994; Shanks et al., 1991). However, no information is available on the effects of stress in 129 strains and no studies have looked at the

effects of chronic restraint stress on changes in neurotransmitters in these two strains. Chronic restraint stress, however, has been shown to affect reaction to neurotransmitter agonists and antagonists such as amphetamine (Badiani et al., 1992), apomorphine (Puglisi-Allegra et al., 1990), oxotremorine (Pullia et al., 1996), ethanol (Boone, Cook, Hou, & Jones, 1997) and anesthetic drugs (Homanics, Quinlan, & Firestone, 1999), suggesting differences in these systems. Chronic restraint stress also induces sensitization to amnesic affects of scopolamine (Zerbib et al., 1990). In addition, while strain differences in reactivity to stress exist, it is unclear whether differences in baseline neurotransmitter levels exist and whether these differences will be associated with differences in the stress response.

The present study characterized and compared two mouse strains widely used in the generation of null mutants, the C57BL/6 and 129/SvEvTac strains. Overall activity was measured on the open field, and learning and memory was assessed with the object recognition and radial arm maze tasks. Furthermore, the effects of chronic stress, an environmental influence, on activity and learning and memory performance were examined. Finally, mice were sacrificed after behavioral analysis and levels of monoamines and metabolites in brain areas contributing to learning and memory were measured in control and stressed subjects of both strains. This study provides insight into the role and interaction of genes (specific to 129/SvEvTac and C57BL/6 mouse strains) and environment (stress) in relation to an important CNS function (memory).

EXPERIMENTAL PROCEUDRES

Subjects

Thirty-two mice served as subjects. Twelve C57BL/6J and twenty 129/SvEvTac mice (5-6 weeks old), in two cohorts, were obtained from Taconic Farms (Germantown, NY) and were acclimated for one week in Hunter College's animal facility before testing began. A smaller number of C57BL/6J subjects were included in this study because pilot data obtained in this lab had already established robust baseline performance of these subjects. Both cohorts in this experiment were exposed to the same conditions, and statistical analysis confirmed no differences between cohorts on behavioral measures. In both cohorts, half the mice from each strain (total of 6 C57BL/6J and 10 129/SvEvTac) underwent 14 days of chronic restraint stress that were for two 2-hour periods daily in Broome rodent restrainers (diameter 25.4 mm). The twice daily stress periods were separated by an hour (in a paradigm similar to that previously described (Zerbib et al., 1990)). Stressed subjects were restrained in a separate room in a sound attenuated box during the 4 hours of stress to diminish effects of any vocalizations or pheromones on non-stressed controls. Stressed subjects were weighed daily after the stress period. Non-stressed controls remained in their home cages and were handled and weighed daily. Subject's weights were analyzed across the stress period with a mixed design, repeated measures ANOVA to assess strain (C57BL/6J vs. 129/SvEvTac), stress (control vs. stress), stress day (0 – 14 day), and interaction effects.

Subjects were maintained on a 12:12 reverse light/dark cycle (lights off 9 AM) and tested during the subjective active period starting at 10 AM. Subjects

were double housed during acclimation, stress, open field testing, and singly housed during object recognition testing and radial arm maze testing. Subjects were sacrificed during a delay period on the radial arm maze in a separate room through rapid decapitation. Their brains were rapidly removed and stored on dry ice for later neurochemical analysis. For all behavioral and neurochemical analysis, the primary investigator had a master list of genotype and stress conditions. Others who assisted in gathering behavioral and neurochemical data were blind to condition.

Behavior Testing

All behavioral testing was done during the subjective active period under dim red light conditions. Subjects were tested on the open field the day following the last stress session. Subjects were then trained on the radial arm maze for ten training/ acquisition trials, followed by seven testing trials, and 4 delay trials of increasing inter-trial duration. Only one trial was run per day. At the end of radial arm maze testing, subjects were tested on the object recognition task. Statistical analyses were performed with NCSS 2000 (Hintze, 1999).

Open Field

On the first day following the 14-day stress regimen, subjects were placed on a 45 cm. square Plexiglas open field with a 4 x 4 grid for 6 minutes. The behaviors recorded were sector visits, rears, grooms, and defecations. A sector visit was defined as a subject bringing at least half of its torso into a sector (no subject can be in 2 sectors at once). Inside sector crossings consist of sector crossings into the middle 4 sectors. Perimeter sector crossings were the 12 sectors

next to the walls of the open field. Rearing was defined as a subject raising its upper torso, so that its forelimbs were at least at the position of its head during ambulation. Wall climbs involved the subject raising its torso and placing its front forelimbs on the wall.

Open field data were analyzed using between subjects 2 x 2 ANOVAS to assess differences and interactions between strains (C57BL/6J vs. 129/SvEvTac) and stress (non-stress vs. stress). Fisher's LSD post-hoc analysis was used where appropriate.

Object Recognition

Following testing on the radial arm maze, object recognition testing began. Subjects were placed on the 45-cm square Plexiglas field with two identical objects for 5 minutes (T1). Exploration of the objects was timed when the subject sniffed at, whisked at, or looked at the objects from no more than 2 cm away. After initial exploration of the identical objects (T1), the subject was removed from the field for an inter-trial delay period. During T1, the time spent exploring the 2 objects was recorded with a stopwatch. After the delay period, one of the objects was removed and replaced with a new object. The time spent exploring the new (novel) and old objects post-delay was recorded (T2), see (Beck et al., 1998) for further details. Pairs of objects used were determined previously to be equally attractive to mice and included button covers, small paper binders, sake cups (porcelain and plastic) and small plastic teacups. The object that served as the old object and the position (left or right) were counterbalanced across groups on each trial and across trials. No object was used for more than one trial.

A trial with a 10-minute inter-trial delay was used to familiarize and acclimate subjects to presence of objects in the field. Both strains, whether stressed or non-stressed, spent more time exploring the novel object than the old object, indicating both strains were able to perform this task (Figure 5A and 6A).

A trial with a 1-hour inter-trial delay assessed object recognition performance on the day following the 10-minute delay trial. A transformation using the arcsine of the square root normalized the percentage of time spent with the novel object (Sokal & Rohlf, 1995). This transformed percentage and the initial time spent exploring the old objects (T1) were analyzed using between subjects 2 x 2 ANOVAS to assess differences and interactions between strains (C57BL/6J vs. 129/SvEvTac) and stress (non-stress vs. stress). Fisher's LSD post-hoc analyses were used where appropriate.

Radial Arm Maze

Twenty-four hours prior to testing on the radial arm maze, subjects were food deprived to 90-95% baseline weight and maintained at this level for the duration of testing on the RAM. Subjects were checked daily for dehydration or lethargy, and more food was given if large weight losses were detected. The eight arms of the maze were each 37.3 cm long and 7.7 cm wide with 9.5 cm high walls. Tops covered each arm but not the center. The center was octagonal with a 23 cm radius. The Plexiglas maze sat on a table which was 74.0 cm off the ground. A number of extra-maze cues were available including a ball of yellow string, a red and white soda can, a green tissue box, two lamps, and black and white checkered wallpaper.

Ten training/ acquisition trials began the day after open field testing. During the 10 acquisition trials, the subject was placed in the center of the eight-arm maze to explore the arms and find the food reward (peanut piece) which was placed at the end of the arm and at 1/3 and 2/3 of the length of the arm. Only one trial was run per day. The subject explored the maze for 10 minutes or until all eight arms had been visited. A visit to the arm was recorded if the subject traveled more than half-way down the arm. The total time to complete the task, total errors (visiting an arm already visited), errors in the first eight choices, and at what choice the first error occurred were recorded for the last 7 of the 10 training/acquisition trials.

Immediately following the training/acquisition trials, 7 testing trials were run. The same procedure was used during training and testing trials except the food reward was only placed at the end of the arm behind a short barrier to prevent the subject from seeing the food until he reached the end of the arm.

After the testing trials, delay trials were run in which the subject was taken off the maze between the 4^{th} and 5^{th} choice for inter-trial delays of 10 minutes, 1 hour, 2 hours, and 3 hours. After the delay period, the subject was placed back on the maze until they visited all the previously unvisited arms. The total number of errors post-delay was recorded. Throughout testing on the radial arm maze, subjects were monitored for development of a response pattern, either sequencing or perseverating. No subjects displayed these patterns on any trials and no trials were therefore eliminated from analysis for this reason. Three subjects, two 129/SvEvTac non-stressed and one 129/SvEvTac stressed subjects, failed to

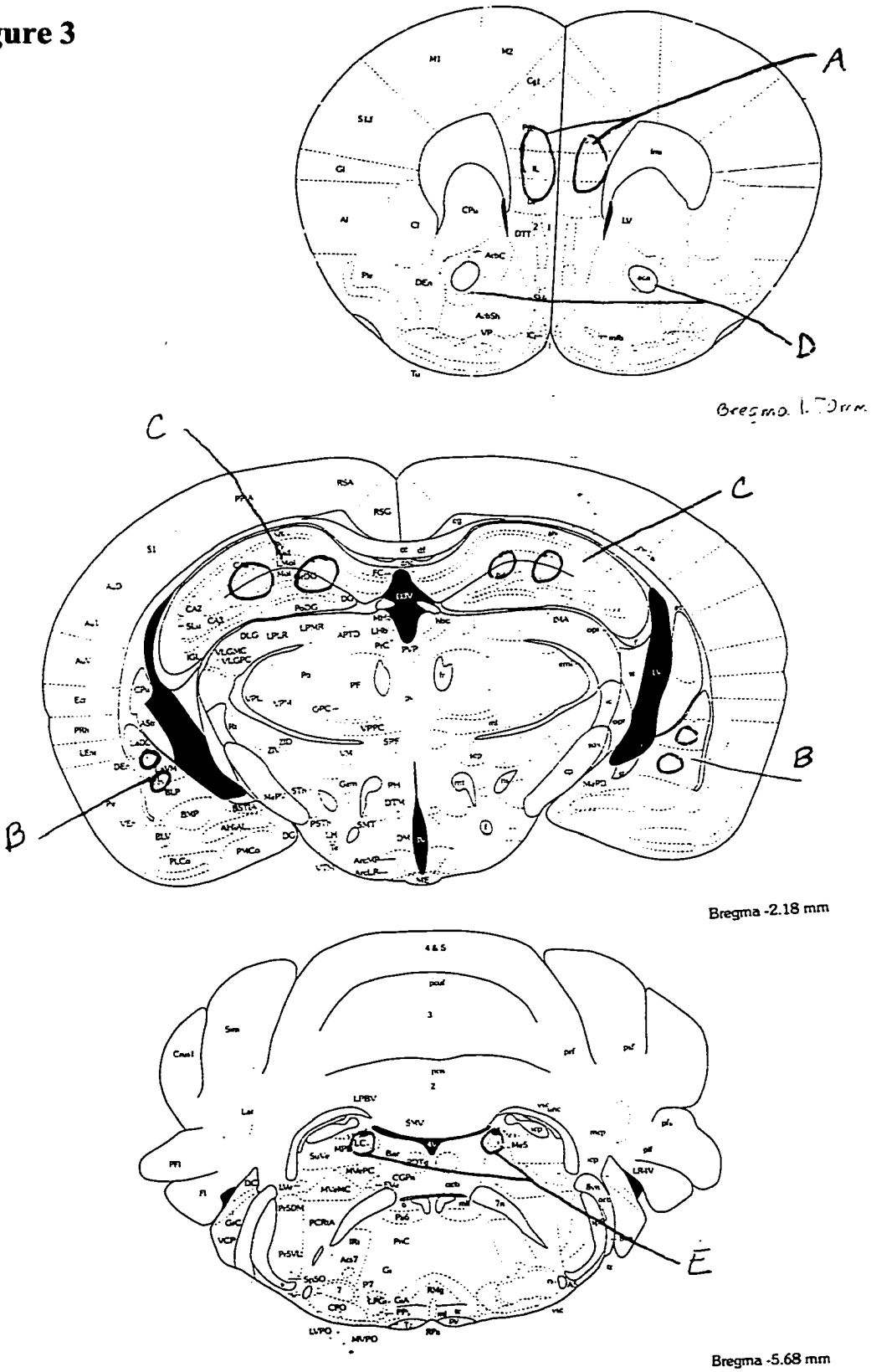
explore the radial arm maze (made fewer than 3 arm choices in 20 to 30 minutes) and were eliminated from the experiment.

Radial arm maze data was analyzed using a mixed design 2 x 2 x 7 (acquisition trials 4-10 or testing trials 1-7) or 4 (delay periods) ANOVA to assess differences and interactions between strains (C57BL/6J vs. 129/SvEvTac), stress (non-stress vs. stress), and across the repeated factor of either trials or delay periods. Fisher's LSD post-hoc analyses were used where appropriate.

Neurochemical analysis

Following completion of all behavioral testing, subjects were placed on the radial arm maze for four arm choices. Immediately following the four arm choices, each subject was taken into a separate room and sacrificed by rapid decapitation (without anesthesia). The brains were quickly removed and immediately placed on dry ice as previously described (Beck et al., 1998; Luine, Bowling, & Hearns, 1990). Brains were serially sectioned at approximately 250μm in a –6 to -8 C in a microtome cryostat. Hippocampus, amygdala, pre-frontal cortex, locus coeruleus, and nucleus accumbens (Figure 3) were micropunched from sliced brain sections according to the atlas of Franklin and Paxinos with a 500μm diameter cannula while the slide rested on a microscope stage maintained at –11.5 C as previously described by (Gogos et al., 1998). Punched samples were placed in 1 ml Eppendorf tubes with 60μl of sodium acetate buffer containing α-methyl-dopamine (as internal standard). After freeze-thawing and centrifugation, the supernatant was removed and 2μl of 1 mg/ml ascorbate oxidase solution (Sigma) was added to each sample to minimize the

Figure 3



HPLC front. Forty microliters was injected in a Waters Associates chromatographic system consisting of a 717 Plus autosampler, 590 pump, and C-18 reverse-phase 3-μm Velosep column (Rainin Instruments). An ESA 5011 Coulocomb 3100A electrochemical detector with the screening electrode set at +0.05 V and the detecting electrode at +0.35 V was used. Concentrations of neurotransmitters and metabolites were calculated by reference to standards using peak integration with a computer assisted Waters Millenium system. Sample runs averaged 30 to 40 minutes. The pellet was then dissolved in 100μl of 0.2 N NaOH for protein determination by the Bradford method (Bradford, 1976). Concentrations of neurotransmitter in each of the brain regions mentioned above were analyzed using between subjects 2 x 2 ANOVAS to assess differences and interactions between strains (C57BL/6J vs. 129/SvEvTac) and stress (non-stress vs. stress). Fisher's LSD post-hoc analyses were used where appropriate.

RESULTS

Weight

There were no significant strain differences in weight across the 14-day stress period, $F_{(4,112)}=1.04$, $p>0.05$. Repeated measures ANOVA of strain and stress effects, revealed stressed subjects of both strains maintained significantly lower weight over the 14 day stress period, $F_{(4,112)}=11.81$, $p<0.0001$, Figure 4.

Open Field

Significant strain and stress differences on the open field were found (Table 2). C57BL/6J subjects had significantly more sector crossings overall ($F_{(1,28)}=20.01$, $p<0.001$), more inside sector crossings ($F_{(1,28)}=26.04$, $p<0.0001$),

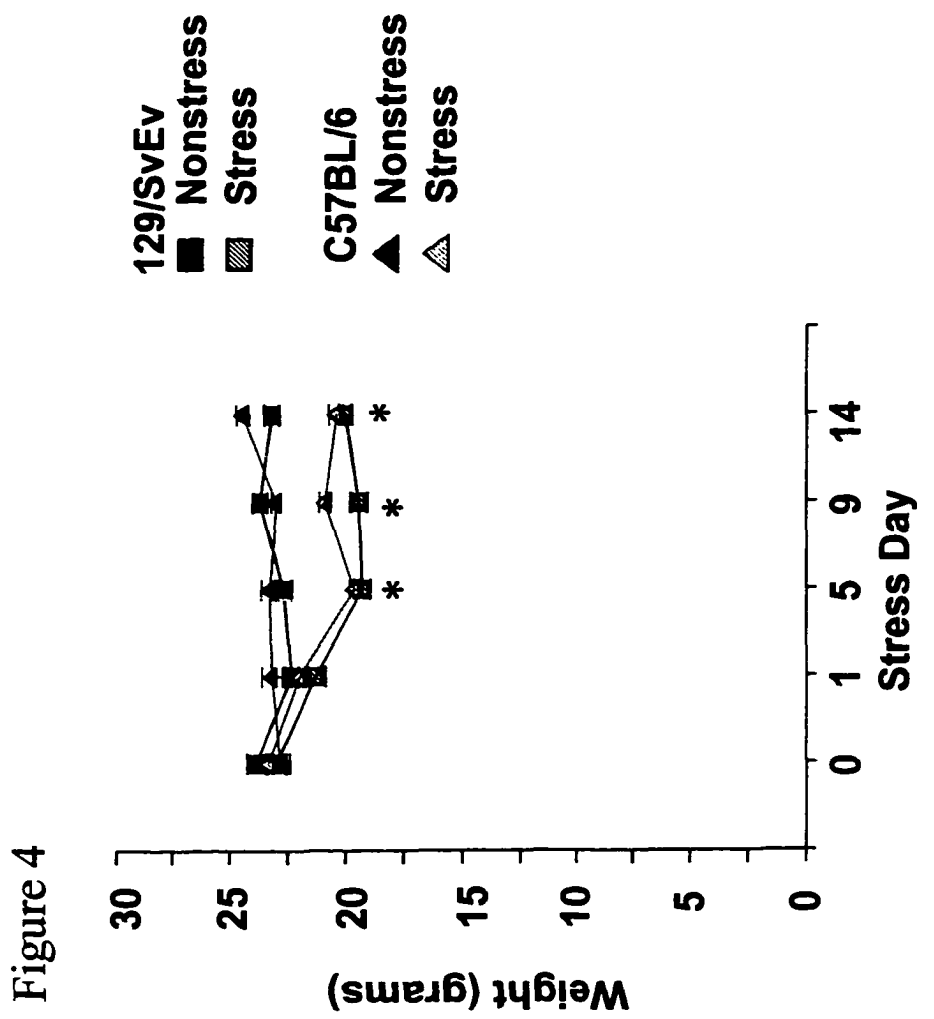


Figure 4

more perimeter sector crossings ($F_{(1,28)}$=11.93, p<0.001), a higher ratio of inside visits to perimeter visits ($F_{(1,27)}$=15.80, p<0.001), more wall climbs ($F_{(1,28)}$=119.62, p<0.001) and fewer defecations ($F_{(1,28)}$=18.35, p<0.001) than 129/SvEvTac subjects (Table 2). A significant interaction between stress and strain existed on total sector crossings ($F_{(1,28)}$=4.77, p<0.05), inside sector crossings ($F_{(1,28)}$=5.20, p<0.05), the ratio of inside crossings to perimeter crossings ($F_{(1,27)}$=6.48, p<0.05), and grooms ($F_{(1,28)}$=6.65, p<0.05). Post-hoc analyses with Fischer's LSD (p<0.016) of the strain by stress interactions revealed that non-stressed 129/SvEvTac subjects had significantly fewer total sector crossings, fewer inside sector crossings, fewer grooms, and a lower inside crossing to perimeter crossing ration than other groups (Table 2).

Object Recognition

Analysis of the amount of time spent with the old objects (T1, sample trial), indicated that C57BL/6J subjects spent more time exploring the objects than 129/SvEvTac subjects before the 10 minute delay (Figure 5A) ($F_{(1,28)}$=8.38, p<0.05) and the 1-hour delay (Figure 5B) ($F_{(1,25)}$=6.03, p<0.05), but not the 3 hour delay (Figure 5C) ($F_{(1,12)}$=0.10, p>0.05). Because of the significant difference in T1, analysis of the percent time exploring the novel object was initially run using T1 as a covariate to adjust for possible effects of initial exploration time on recognition. The analysis did not find T1 to be a significant covariate and therefore a two-way ANOVA was run on the percent time data alone. With a delay of one hour between the sample and recognition trial, C57BL/6J subjects overall spent a smaller percentage of time with the novel

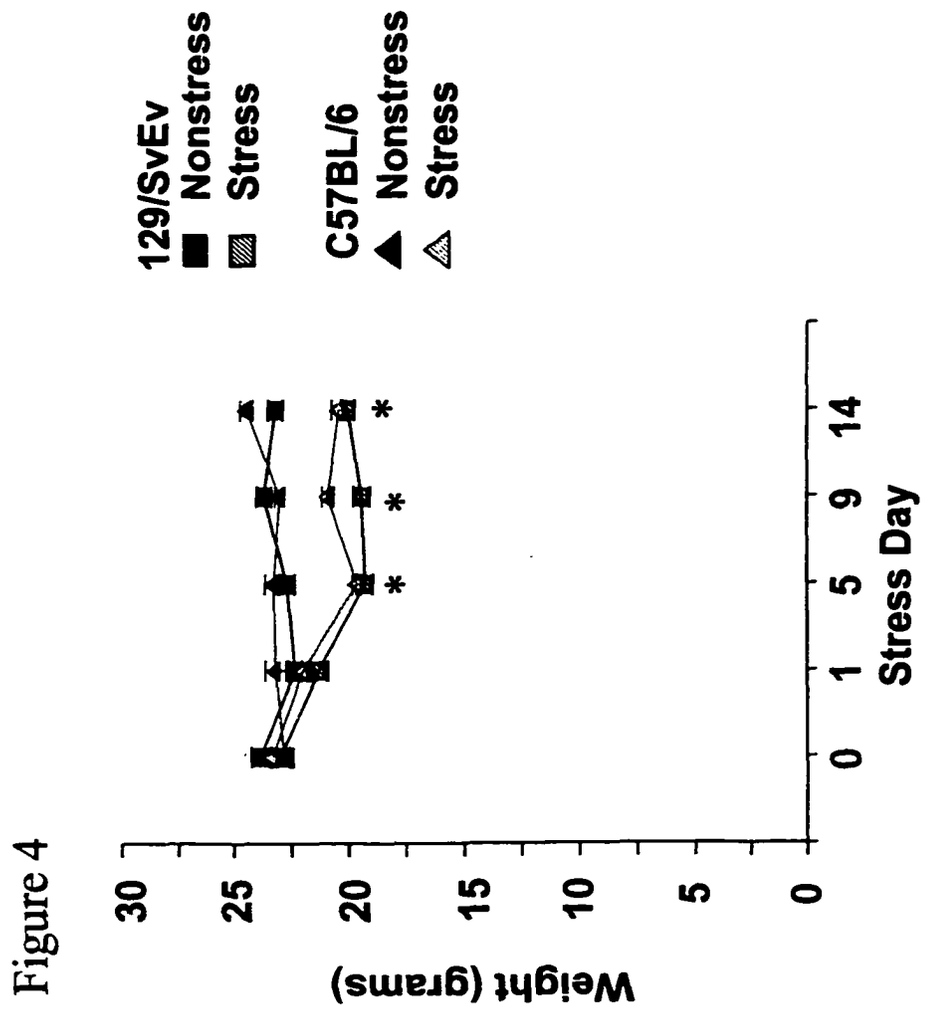


Figure 4

Figure 5

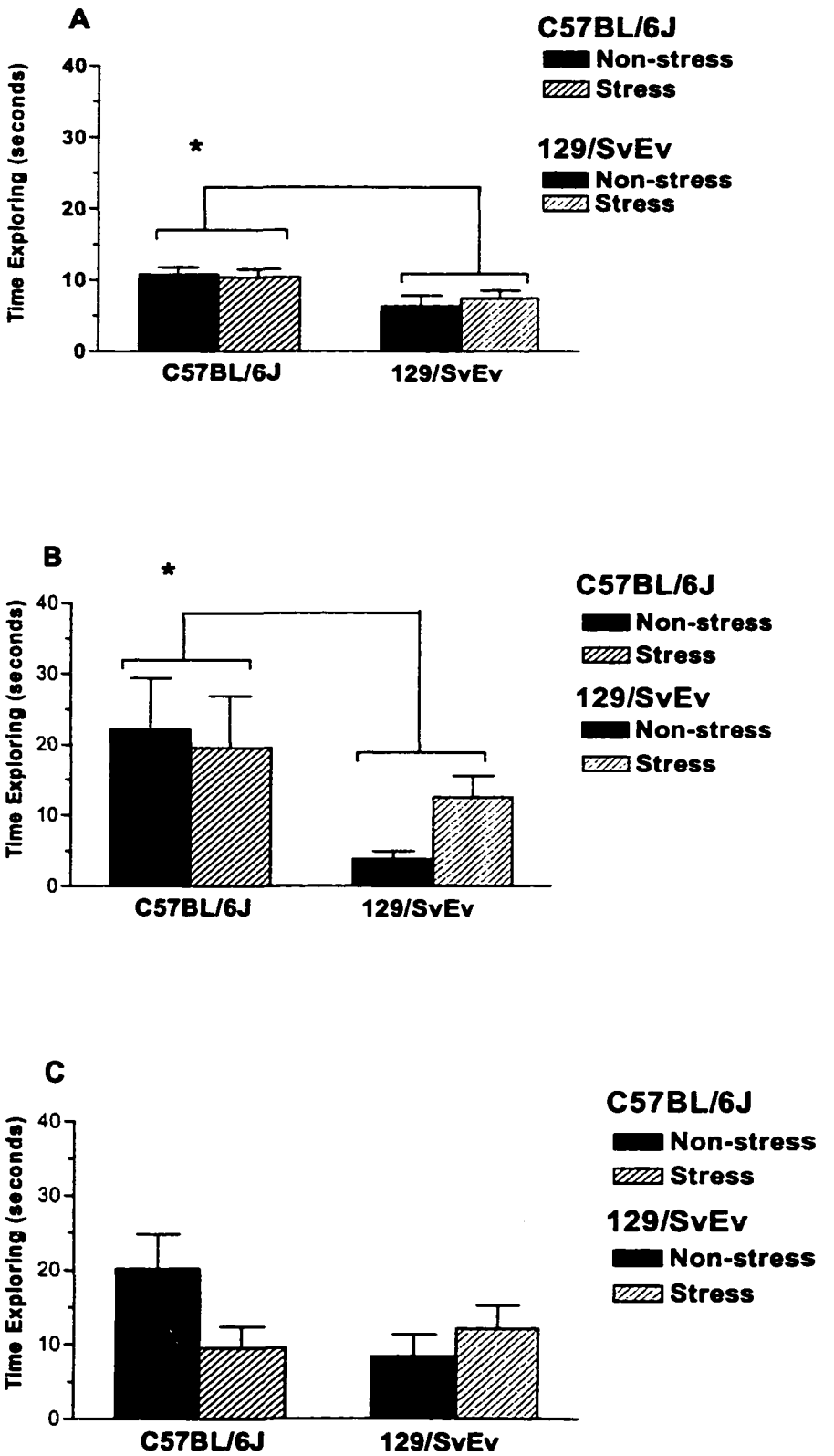
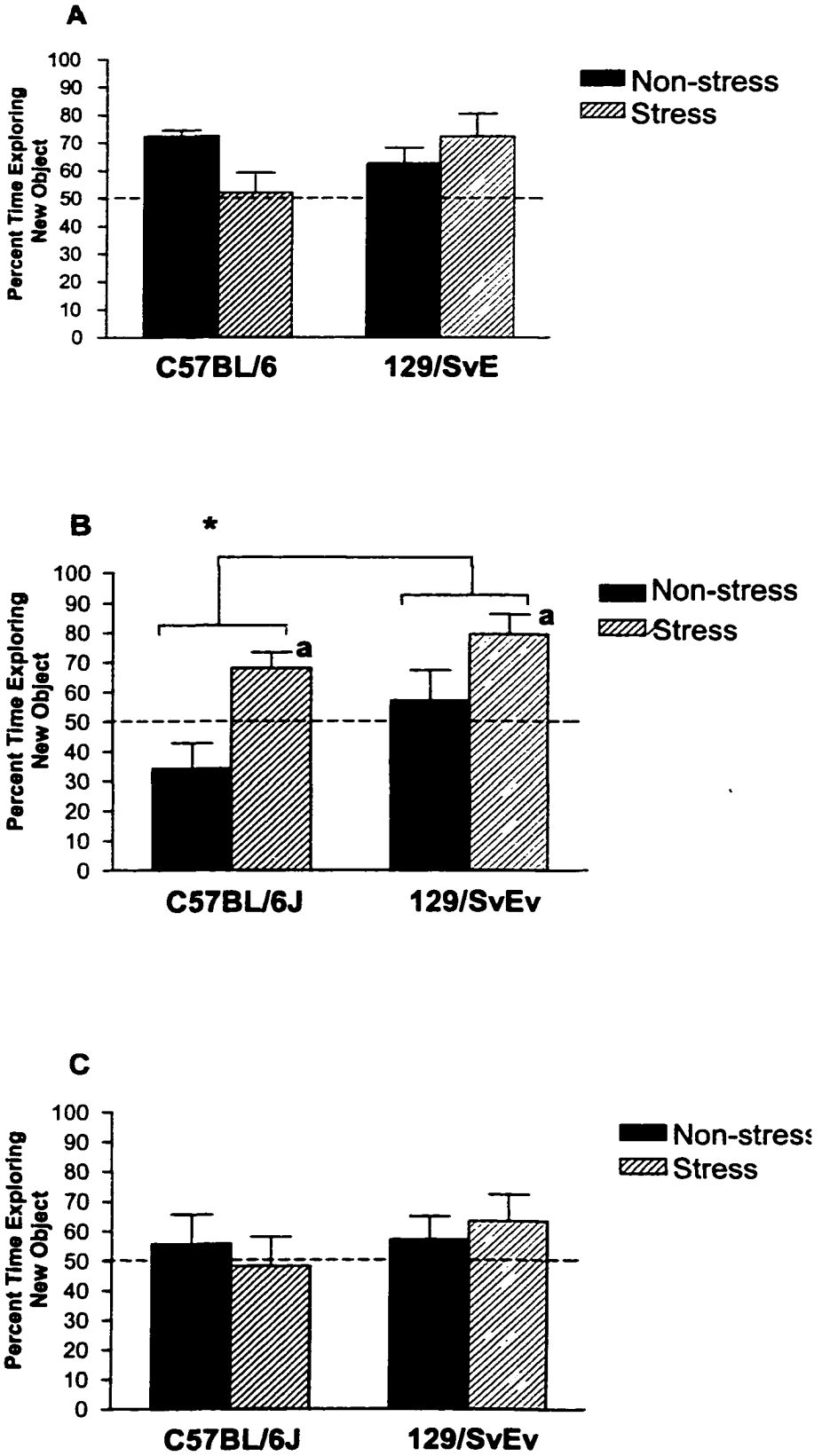


Figure 6



object (strain effect, $F_{(1,25)}$=3.90, p=0.059) than 129/SvEvTac subjects, Figure 6B. Stressed subjects of both strains spent a significantly higher percentage of time with the novel object ($F_{(1,25)}$=8,46, p<0.01) than non-stressed controls, showing an enhancement of performance by stress in both strains Figure 6B.

Radial Arm Maze

Radial Arm Maze Acquisition

There were no significant strain, stress, or interaction effects in 7 acquisition trials of the radial arm maze in the number correct in the first eight choices ($F_{(1, 28)}$=1.15 strain, 0.24 stress, and 0.37 interaction respectively, p>0.05), the number of total errors ($F_{(1, 28)}$=0.39 strain, 0.22 stress, and 0.25 interaction respectively, p>0.05), or the choice of the first error ($F_{(1, 28)}$=2.68 strain, 1.97 stress, and 0.32 interaction, p>0.05 respectively), data not shown.

Radial Arm Maze Testing Trials

C57BL/6J subjects performed significantly better than 129/SvEvTac subjects on radial arm maze testing trials. C57BL/6J subjects spent significantly less time to complete the maze (visit all eight arms) than 129/SvEvTac subjects, $F_{(6,123)}$=4.56, p<0.01, Figure 7. Since there is a significant difference in time to complete the maze, this variable was used as a covariate in the analysis of performance on the radial arm maze trials. With time to complete the maze held as a covariate, a significant strain difference in total errors was found, $F_{(1,198)}$=5.88, p<0.05, Figure 8. C57BL/6J subjects made fewer total errors (5.31 ± 0.33) than 129/SvEvTac subjects (4.12 ± 0.27) Figure 8. Stress did not significantly affect performance on the testing trials in either strain. A significant

difference between the strains was also found in the number correct in the first eight choices, $F_{(1,28)}=4.95$, $p<0.05$, Figure 9.

Radial Arm Maze Delay Trials

During RAM trials with a delay between the 4th and 5th choices, performance of C57BL/6J and 129/SvEvTac subjects was not significantly different. Stress significantly enhanced performance of both groups. Stressed subjects made significantly fewer total errors post-delay than non-stressed subjects, $F_{(1,25)}=5.64$, $p<0.05$, Figure 10.

Neurochemistry

Significant differences in neurochemical levels were found between C57BL/6J and 129/SvEvTac strains in most of the brain areas investigated, Table 3. However, as with behavioral data, stress affected the strains similarly. Levels of norepinephrine (NE) were 7-fold higher in 129/SvEvTac strain compared to C57BL/6J in the nucleus accumbens ($F_{(1,26)}=12.48$, $p<0.01$), 12-fold higher in the locus coeruleus ($F_{(1,26)}=12.48$, $p<0.01$), but in the amygdala 129/SvEvTac had about half the amount of NE compared to C57BL/6J ($F_{(1,26)}=3.69$, $p=0.07$), Table 3. Although no differences in NE levels were found in the frontal cortex, turnover, as measured by the ratio of MHPG to NE was significantly lower in 129/SvEvTac subjects (non-stressed controls = 0.04 ± 0.03) compared to C57BL/6J (non-stressed controls = 0.12 ± 0.04) ($F_{(1,18)}= 4.75$, $p<0.05$), approximately one-third of the turnover present in the C57BL/6J subjects.

Serotonin was also elevated in 129/SvEvTac compared to C57BL/6J in the nucleus accumbens ($F_{(1,26)}=7.36$, $p<0.05$) and frontal cortex ($F_{(1,20)}= 4.64$,

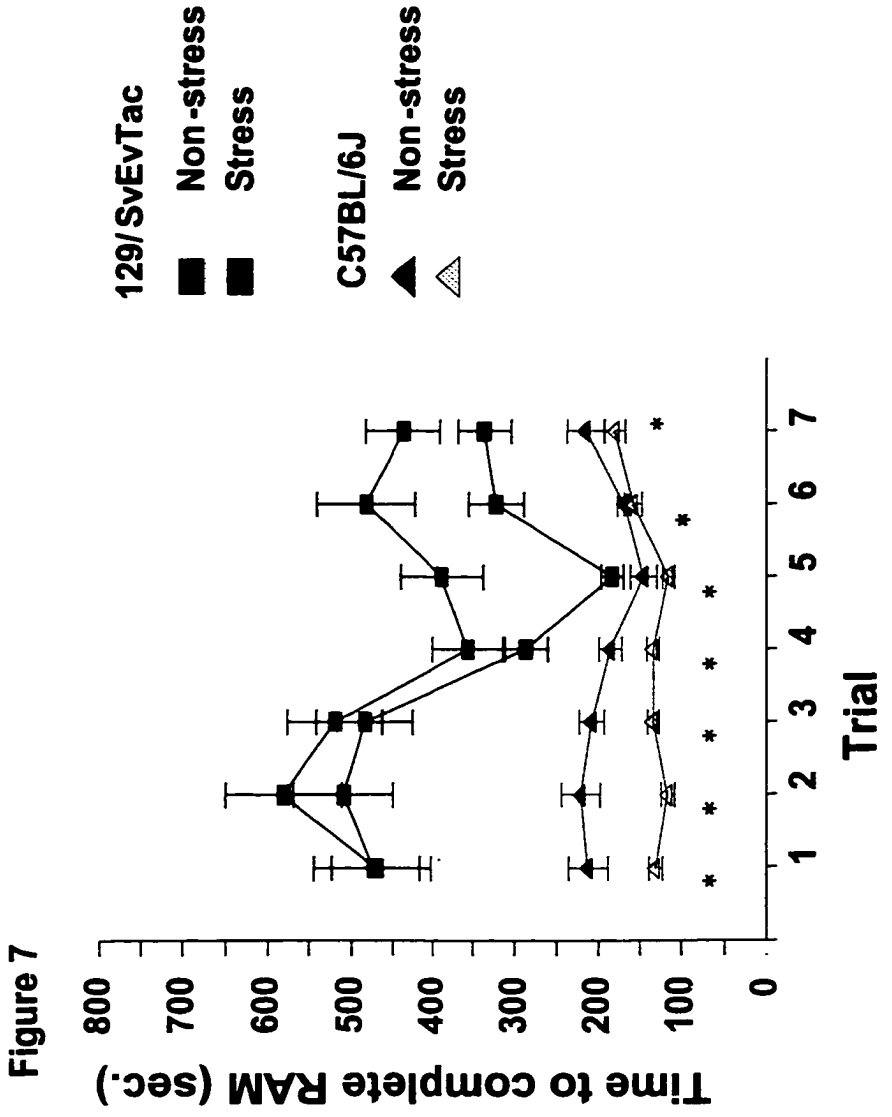
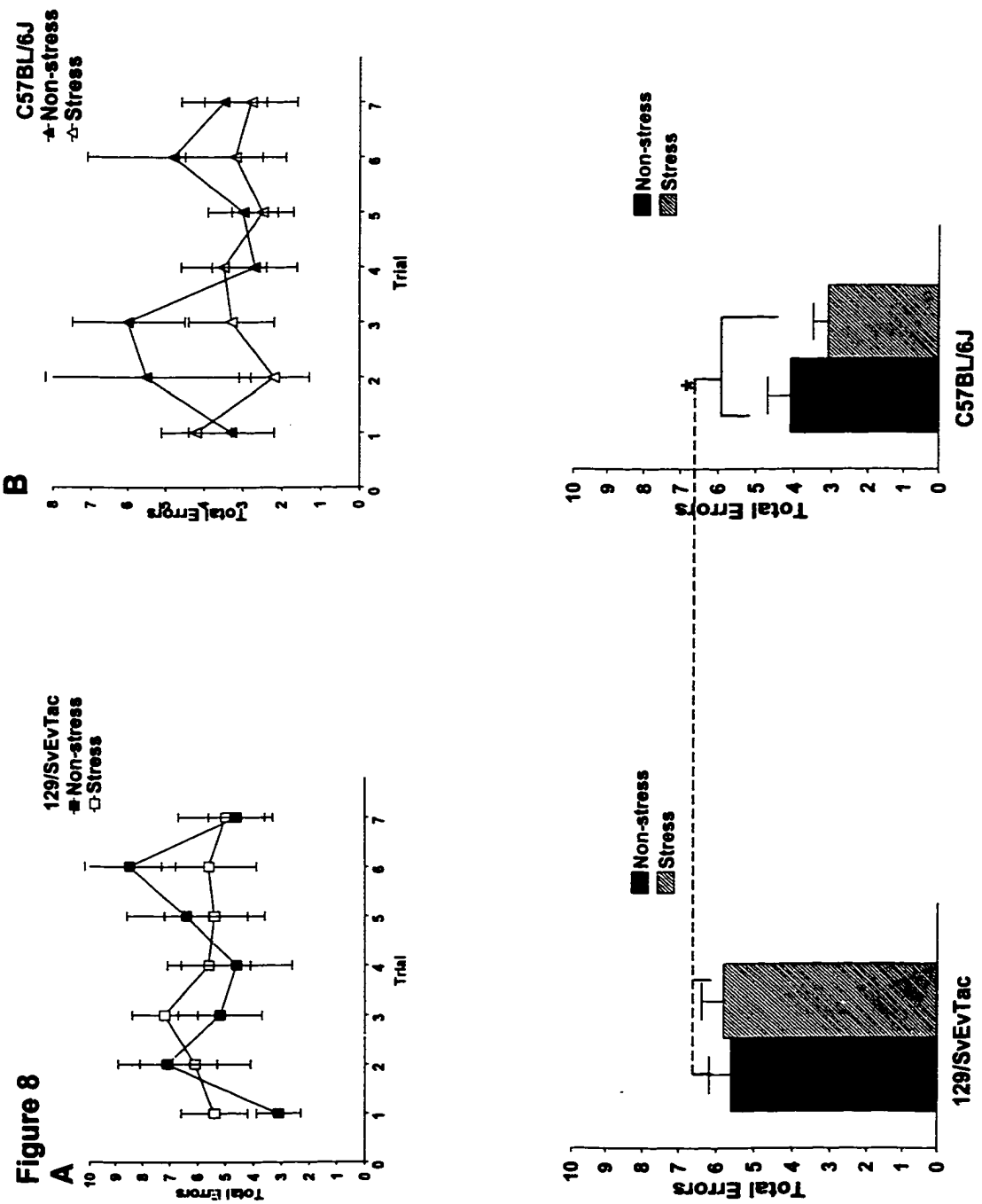


Figure 7

Figure 8



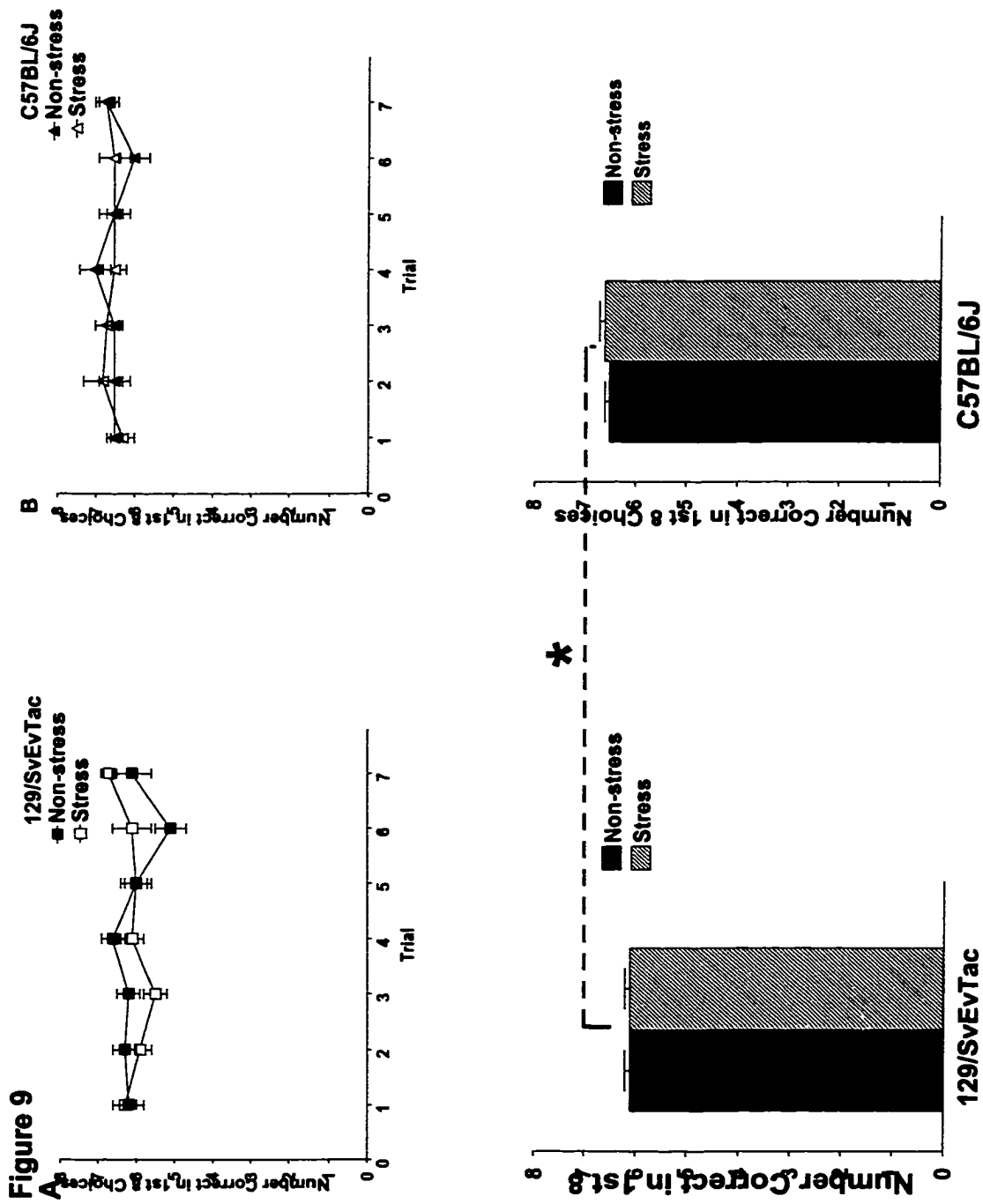


Figure 9

Figure 10

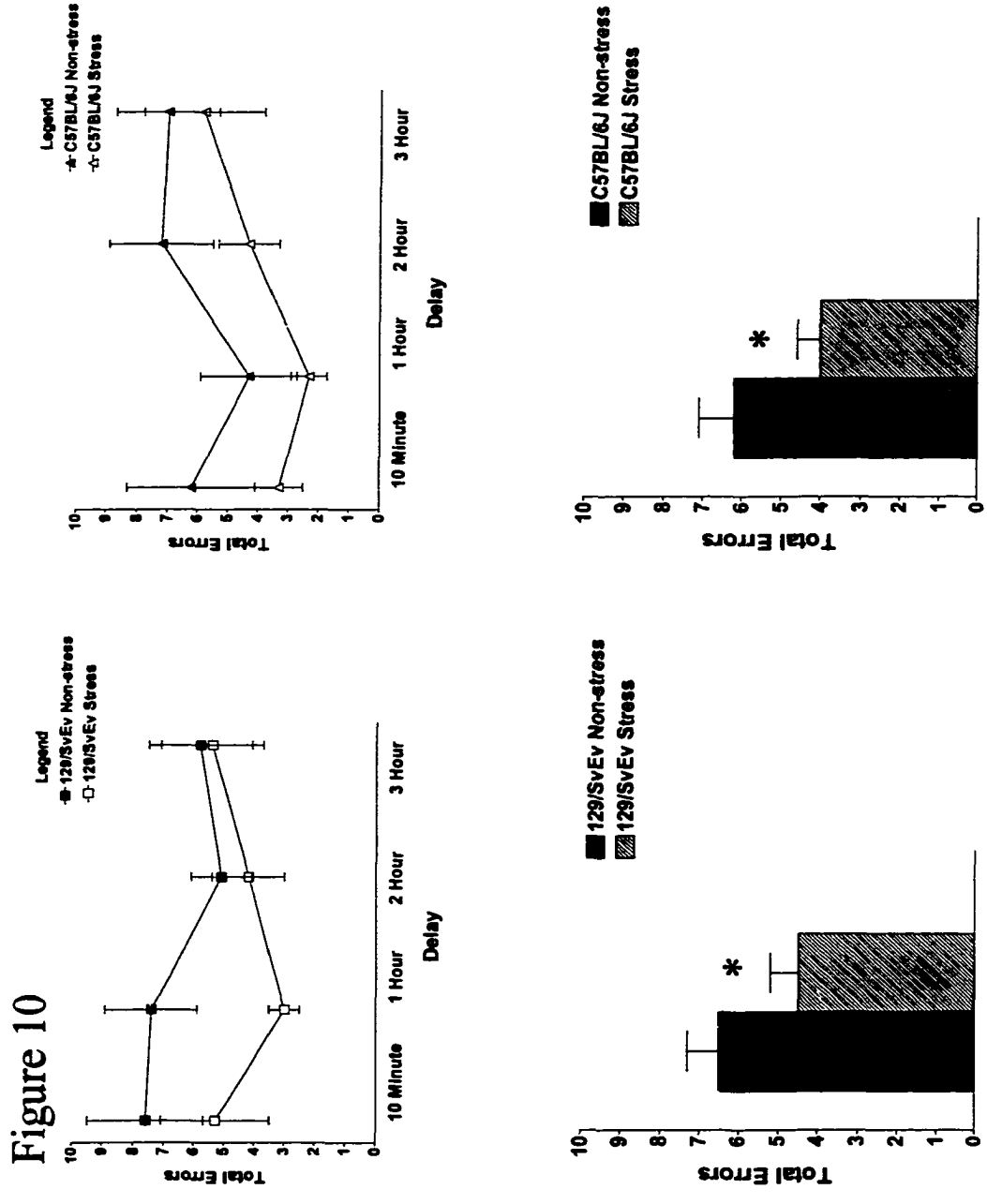


Table 3

Brain Region	Strain	NE	5HT	5HIAA	5HIAA/5HT
Frontal Cortex	C57BL/6J (n=5)	16.8 ± 1.3	2.5 ± 1.0	2.0 ± 0.5	0.58 ± 0.0[a]
	129/SvEvTac (n=8)	13.9 ± 0.8	6.2 ± 0.9*	2.8 ± 0.5	0.46 ± 0.1
Locus Coeruleus	C57BL/6J (n=6)	1.5 ± 0.7	1.4 ± 0.4	12.3 ± 2.9	11.3 ± 3.3
	129/SvEvTac (n=9)	13.1 ± 3.1**	5.7 ± 1.3	18.7 ± 4.8	4.2 ± 0.9*
Nucleus Accumbens	C57BL/6J (n=6)	2.2 ± 1.0	2.7 ± 0.4	28.7 ± 7.3	11.3 ± 3.3
	129/SvEvTac (n=9)	14.1 ± 2.5**	8.0 ± 0.9*	29.9 ± 7.2	4.1 ± 0.8*
Amygdala	C57BL/6J (n=6)	3.3 ± 0.3	4.3 ± 0.8	6.1 ± 1.1	1.5 ± 0.3
	129/SvEvTac (n=9)	1.9 ± 0.3**	2.8 ± 1.1*	6.9 ± 1.6	2.6 ± 0.5*

Mean ± S.E. pg/ μg of protein norepinephrine (NE) or serotonin (5-HT) or ratio of neurotransmitter to metabolite in the frontal cortex, locus coeruleus, amygdala, and nucleus accumbens of non-stressed C57BL/6J and 129/SvEvTac subjects. Significant differences were found in norepinephrine levels in the locus coeruleus (**$p<0.01$), nucleus accumbens (**$p<0.01$), and amygdala (**$p<0.01$). In addition, 5-HT levels and the ratio of 5HIAA to 5-HT were also significantly different between C57BL/6J and 129/SvEvTac strains in the nucleus accumbens (*$p<0.05$) and amygdala (*$p<0.05$). [a]n=3.

$p<0.05$). Turnover of serotonin into 5HIAA reflected differences between brain regions. In the nucleus accumbens, the ratio of 5HIAA to 5HT was significantly lower in 129/SvEvTac than C57BL/6J ($F_{(1,24)}=4.79$, $p<0.05$) and significantly higher in the amygdala ($F_{(1,21)}=4.93$, $p<0.05$) and the locus coeruleus ($F_{(1,24)}=4.79$, $p<0.05$), Table 3.

However, stress did not exert major changes on neurochemical measures in either strain. No significant main effects for stress were found in any brain regions for any monoamine. For serotonin in the nucleus accumbens, the strain and stress interaction was significant ($F_{(1,26)}=4.60$, $p<0.05$) and post-hoc analysis with Fisher's LSD Multiple Comparison post-hoc, $p<0.017$ revealed that non-stressed subjects of the 129/SvEvTac strain had significantly higher levels of serotonin than the other groups (stressed and non-stressed C57BL/6J and stressed 129/SvEvTac), data not shown.

DISCUSSION

The present study has compared memory performance, open field activity, neurochemical levels in several brain regions, and stress reactivity in two strains of inbred mice, C57BL/6J and 129/SvEvTac. Reaction to stress is similar in both strains across memory tasks and in most neurochemical measures. Some differences between strains in baseline memory performance, open field activity, and neurochemical levels exist. In addition, stress does affect locomotor activity differently in the two strains. Strain differences in memory depend on the particular memory task being used to assess performance. The results suggest that conclusions of differences in memory performance be carefully drawn in these

strains or in mice with a mixed background of these strains. In addition, these results suggest that accurate assessment of memory performance should include more than one measure of memory performance. As far as stress effects, however, these results suggest that enhancement in memory performance with 14-days of chronic stress is robust across several memory tasks.

Open Field

Results from the open field reveal that C57BL/6J are generally more active and ambulatory than 129/SvEvTac subjects. There is also evidence of less anxiety in C57BL/6J subjects in measures of the ratio of inside crossings to perimeter crossings, wall climbs, and defecations. These results support findings by Homanics et al. (1999) and Paulus et al. (1999) who compared substrains of 129 mice to C57 subjects and found that the 129 is less active on the open field and may be more anxious. In this study, stress increased activity of 129/SvEvTac to the level of the C57BL/6J on most measures and did not affect activity of C57BL/6J on the open field.

Radial arm maze

Results from the current study confirm significant differences between the C57BL/6J and 129 strains in memory performance (Nguyen et al., 2000; Owen et al., 1997). During testing trials of the radial arm maze, C57BL/6J subjects exhibit fewer total errors than 129/SvEvTac subjects. Though no other studies to date have compared performance of the 129/SvEvTac to the C57BL/6J strain on the radial arm maze directly, other paradigms for assessing spatial memory have found significant differences between these strains (Nguyen et al., 2000; Owen et

al., 1997). Specifically, C57BL/6J subjects exhibit superior performance on the Barnes maze, another spatial and land based memory task (Nguyen et al., 2000). However, studies of strain differences on the spatial Morris water maze have reported that C57BL/6J mice had inferior performance (Nguyen et al., 2000; Owen et al., 1997). Factors contributing to differences among these studies include the types of memory that the tasks assess including working memory on the radial arm maze and Barnes maze versus reference memory on the Morris water maze. In addition, the radial arm and Barnes maze contain an aspect of reinforcement whereas the Morris water maze includes an aversive factor (water). Further, the radial arm and Barnes maze are land-based mazes that require ambulation while the Morris water maze involves swimming. In addition to the above factors, the increase in anxiety-like behaviors displayed by the 129/SvEvTac subjects assessed on the open field may have also contributed to their poorer performance on the radial arm maze and Barnes maze (Lathe, 1996).

Strain differences are no longer apparent in the delay trials of the radial arm maze. Disappearance of strain differences on delay trials of the radial arm maze may be due to reduction in the influence of motor differences and/ or anxiety. It is possible that repeated testing on the radial arm maze reduces differences due to motor impairment. By the delay trials on the radial arm maze, subjects have been exposed to the maze fourteen times for acquisition and testing trials. Recent evidence suggests that repeated testing can eliminate strain differences in motor coordination (Homanics et al., 1999), which may mimic repeated testing on the radial arm maze. In addition this repeated exposure to the

maze may reduce anxiety. Therefore, the delay trials may be a more accurate assessment of differences in memory. Delay trials are considered to be more cognitively demanding (Baimbridge & Miller, 1982), and thus may provide more accurate assessment of memory differences. While no studies have investigated the effects of chronic stress in mice, these findings are consistent with findings in rats that 13 days of chronic restraint stress enhances delay trial performance on the radial arm maze (Luine et al., 1994a).

Stress enhanced performance of both strains when tested with inter-trial delays on the radial arm maze. Delay trials are considered to be more cognitively demanding (Baimbridge et al., 1982), and thus performance enhancement during delay trials may indicate a ceiling effect in non-stressed subjects during non-delay trials which was undetectable.

Object recognition

Although initially all subjects discriminated between the old and new objects on the object recognition task with an inter-trial delay of 10 minutes, extension of the inter-trial delay period to 1-hour on a subsequent trial revealed strain differences. Remarkably, the 129/SvEvTac subjects perform better than the C57BL/6J on this test of non-spatial memory. The 129/SvEvTac subjects spend less time exploring the objects on the field in the sample trial, a result consistent with their higher anxiety level on the open field. Despite lower initial exploration of the objects, the 129/SvEvTac subjects spend a higher percentage of time exploring the novel object during the recognition trial compared to C57BL/6J

subjects. These results suggest better memory in this task by the 129/SvEvTac subjects than C57BL/6J subjects.

Interestingly, these results contrast directly with the finding of better performance of the C57BL/6J subjects on the radial arm maze. Thus, performance on the radial arm maze in C57BL/6J subjects may indicate differences in the types of memory each task assesses. Since the object recognition task relies less on ambulation than the radial arm maze and does not require food restriction, the object recognition task may be a useful tool in assessing memory in mice. In addition, the object recognition task is considered a non-spatial memory task which is disrupted by destruction of the entorhinal cortex while performance on the radial arm maze requires spatial memory and hippocampal function (Ennaceur, Neave, & Aggleton, 1996; Ennaceur, Neave, & Aggleton, 1997; Olton & Papas, 1979). The diverse loci of these types of memory may illuminate strain differences in brain morphology, such as previously reported differences in corpus callosum (Balogh, et al., 1999). These results confirm the need for several tests of memory to accurately assess memory performance in the two strains or in a mixed background.

As with the radial arm maze, both strains were enhanced by stress suggesting that the stress effect is a robust finding which is not dependent on memory task type. In addition, this one-hour delay period on the object recognition task seems optimal for testing stress effects on memory in these strains.

Stress Effects

Stressed subjects failed to gain weight across the stress period, a finding consistent with the effect of stress in rats (Watanabe et al., 1992). On the open field, stress significantly enhanced activity levels in the 129/SvEvTac strain, but did not significantly increase activity in the C57BL/6J subjects which may reflect a ceiling effect in the C57BL/6J suggesting that activity level was at a maximum level already.

Regardless of memory task, stress enhanced both strains. With a one-hour inter-trial delay on the object recognition task, stressed subjects of both strains spent significantly more time exploring the novel objects. Additionally, stress enhanced performance of both strains on delay trials of the radial arm maze. This study is the first to compare the effects of chronic restraint stress on learning and memory in mice. Results presented here in mice are consistent with findings in rats that approximately 2 weeks of restraint stress enhanced memory performance on the radial arm maze (Luine et al., 1996). Additionally, no significant changes in neurochemistry were found. Lack of changes after stress is probably attributable to the length of time after stress in which the brains were extracted and measured. After behavior testing on both the radial arm maze and object recognition tasks, approximately 2 and a half weeks had passed. Previous reports have shown transient stress effects in rats several weeks following stressor onset (Luine et al., 1994a; Watanabe et al., 1992).

Neurotransmitter Levels

Significant strain differences exist in neurochemical levels in several brain regions involved in learning, memory, and emotional reactivity. In the locus coeruleus, nucleus accumbens, and amygdala, serotonin levels and serotonin turnover were significantly different between the two strains. High levels of 5-HT have previously been correlated with diminished performance on the radial arm maze (Luine et al., 1994a). This study has found that the 129/SvEvTac subjects have higher levels of 5-HT and lower levels of turnover in most of the brain areas investigated in this study and exhibit significantly lower performance on the radial arm maze compared to the C57BL/6J subjects. Thus, in this study, the strain with high levels of 5-HT also has worse performance on the radial arm maze, supporting the findings of Luine et al. (Luine et al., 1994a).

Rossi-Arnaud and Ammassari-Teule (1998) (Rossi-Arnaud & Ammassari-Teule, 1998) have proposed that strain differences in learning and memory may be due to differences in neural connections subserving memory performance. While no studies comparing connections between C57BL and 129 strains are available, differences between C57BL and other strains have been reported. For instance, in addition to the hippocampus which is important in performance of spatial memory tasks, connections to the frontal cortex and amygdala may be important for C57BL/6J strains whereas connections to the nucleus accumbens may be essential for other strains such as DBA (Rossi-Arnaud et al., 1998). In addition, significant reductions in corpus collosum size and connectivity have been reported for 129 strains compared to other strains (Balogh et al., 1999).

Though decreases in corpus collosum size were not correlated with impaired memory performance (Balogh, et al., 1999), it is apparent that significant differences between strains exist in central nervous system functioning and structure. Our findings suggest large differences in neurochemical levels which may reflect differences in morphology.

Differences between the strains in NE or NE turnover in the locus coeruleus and frontal cortex may affect neural signaling and particularly attention aspects of behavior which may, in turn, contribute to memory function (Skosnik, Chatterton, Jr., Swisher, & Park, 2000). Increased NE in the locus coeruleus and decreased turnover of NE in the frontal cortex of 129/SvEvTac subjects may explain their enhanced ability to discriminate between the old and new objects in the object recognition task, which involves attention components (Downing, 2000). Further, stressed subjects of both strains were able to discriminate better than controls between the old and new objects at a one hour delay on the object recognition task. The stressed subjects had decreased levels of NE in the frontal cortex as compared to controls. This result suggests that changes in NE, particularly in the frontal cortex, can influence attention and thus, object recognition performance (Birnbaum, Gobeske, Auerbach, Taylor, & Arnsten, 1999). Changes in NE in the frontal cortex have been previously reported as a result of both chronic and acute stress (Shanks et al., 1991; Kneavel, Christakos, Ferguson, & Luine, 2000).

Conclusions

This study has highlighted clear distinctions in performance on learning and memory tasks between inbred mouse strains. These strain differences are task dependent and coexist with large differences in neurochemistry which may reflect neuroanatomical differences. Thus selection of a particular strain for tests of memory performance should take into account these results. In addition, these results suggest that the influence or contribution of parental background strain be taken into account when assessing the phenotype of null mutants or transgenic models. However, similarities in stress response on both spatial and non-spatial memory tasks suggest that both strains and chimeras derived from cross breeding of these strains may be useful for studying stress effects on cognitive function.

Chapter 3: Effect of chronic stress in calbindin-D_{28k} null mutant and wild-type subjects on spatial and non-spatial memory tasks.

INTRODUCTION

Stress is known to be an environmental force that alters the homeostasis of an organism and leads to a 'fight or flight' response (Cannon, 1935). This response is a result of activation of the sympathetic nervous system and increased arousal of the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al., 1997), (Jacobson et al., 1991). Chronic stress results in prolonged activation of the sympathetic nervous system and is associated with behavioral and physiological changes. If stress persists, these changes can result in a variety of transitory effects including decreased immune system functioning (Yin, Tuthill, Mufson, & Shi, 2000), alterations in neurochemistry (Beck et al., 1998), and behavioral changes (e.g. (Weiss, Glazer, Pohorecky, Brick, & Miller, 1975; Beck et al., 1998)). Tasks requiring an animal to learn or remember specific, adaptive responses to stimuli are particularly vulnerable to the effects of chronic stress (Luine et al., 1994a; Luine et al., 1996).

Calbindin-D_{28K} is an important central nervous system regulatory protein that is expressed in brain regions along the HPA axis (Iacopino, Rhoten, & Christakos, 1990; Lephart, 1996; Abe, Amano, Yamakuni, Takahashi, & Kondo, 1990; Lephart, Watson, Jacobson, Rhees, & Ladle, 1997). While it is not clear what the role of calbindin-D_{28k} is in the stress response, evidence indicates that alterations in calbindin-D_{28k} expression are associated with stress. Specifically,

calbindin-D_{28k} may protect cells from increases in excitatory amino acids that result from release of the stress hormone, corticosterone (Jouvenceau et al., 1999). CA1 and CA2 subfields of the hippocampus, which contain calbindin-D_{28k}, are resistant to neuronal insult from both corticosterone (Iacopino & Christakos, 1990a) and chronic stress (Krugers, Koolhaas, Medema, & Korf, 1996). In addition, calbindin-D_{28k} levels increase in these cells in response to chronic stress, suggesting that alterations in calbindin-D_{28k} expression are responsible for protection in these neurons (Krugers et al., 1996).

The protective action of calbindin-D_{28k} may be related to its ability to buffer or reduce intracellular calcium (Ca^{2+}) levels (Mattson, Rychlik, Chu, & Christakos, 1991). This buffering or reduction of Ca^{2+} protects the cell from Ca^{2+} mediated cell death. Calbindin-D_{28K} positive areas of the brain, including, CA1, CA2, and dentate gyrus areas of the hippocampus, the forebrain, the nucleus basalis, and the cerebellum, are resistant to calcium mediated cell death due to ischemic injury, glutamate damage, and calcium ionophore damage (Schwob, Fuller, Price, & Olney, 1980; Mattson et al., 1991). Additionally, introduction of exogenous calbindin-D_{28K} in neuronal cell cultures renders these cells more resistant to degeneration by a variety of insults and toxins including dexamethasone, immunoglobulin G (IgG) from Amyotrophic Lateral Sclerosis (ALS) patients, and β-amyloid found in plaques of Alzheimer's patients (Dowd, MacDonald, Komm, Haussler, & Miesfeld, 1992; Ho et al., 1996; Prehn et al., 1996) (Guo, Christakos, Robinson, & Mattson, 1998). Chard et al. (1993) suggest that this protective effect may be due to reduction in the rate of rise of

intracellular calcium levels by calbindin-D_{28k} that occurs with depolarization of the cell.

Calbindin-D_{28k} has also been shown to be associated with sexual differentiation of brain regions linked to the HPA axis including preoptic area (Sickel & McCarthy, 2000), hypothalamus (Brager, Sickel, & McCarthy, 2000; Taylor, Quintero, Iacopino, & Lephart, 1999; Watson, Taylor, & Lephart, 1998; Lephart et al., 1997; Lephart, 1996), dentate gyrus (Tanapat, Hastings, Reeves, & Gould, 1999), pineal body (Bastianelli E & Pochet, 1993), and pituitary (Abe et al., 1990). Thus, differences in expression of calbindin-D_{28k} during development are associated with sexual differentiation of several brain regions including regions that are susceptible to stress effects and regions involved in learning and memory (Sickel et al., 2000; Brager et al., 2000; Tanapat et al. 1999). However, no studies have investigated calbindin-D_{28K} in relation to possible sex differences in learning and memory or to sex differences in response to stress.

The importance of examining the role of calbindin-D_{28k} in learning and memory using a stress manipulation can be seen. Stress alters calbindin-D_{28k} expression and this alteration can affect learning and memory performance as well as brain neurochemistry (Iacopino et al., 1990a). In addition, several hypotheses suggest that disease vulnerability increases with increased exposure to stress (Zerbib et al., 1990). Thus disease vulnerability related to calbindin-D_{28k} may be increased or affected by chronic stress. Some evidence suggests that Alzheimer's disease (AD), a neurological disorder affecting memory function, may be related to changes in calbindin-D_{28k} levels. Accumulation of β-amyloid,

which comprises neuritic plaques related to cognitive deficits in AD patients, results in neuronal degeneration see Dumery et al. (2001) for review. This degeneration is attenuated by calbindin-D_{28k} (Dowd et al., 1992). Unfortunately, cells in areas of the brain containing calbindin-D_{28K} are highly degenerated in Alzheimer's patients, thus potentially resulting in decreased protection from calbindin-D_{28K} (Price et al., 1982; Ichimiya, Emson, Mountjoy, Lawson, & Heizmann, 1988; Iacopino & Christakos, 1990b). This loss of calbindin-containing cells is accompanied by changes in calcium homeostasis which has been hypothesized to be involved in increased cell death and the pathogenesis related to AD (Nishiyama, Ohwada, Iwamoto, & Arai, 1993).

Recent creation of a calbindin-D_{28k} null mutant mouse (cal -/-) is useful for studying the role of calbindin-D28k under vulnerable central nervous system conditions. Specifically, deletion of calbindin-D_{28k} allows for *in vivo* clarification of the role of calbindin-D_{28K} in the stress repsonse as well as learning and memory function. The mechanisms by which learning and memory functions are altered by stress and how these functions are mediated by sex differences in the cal -/- mouse will begin to clarify calbindin-D_{28K} mechanisms of action. Previous studies with calbindin-D_{28k} deficient mice have reported no general developmental or nervous system structure changes (Gary, Sooy, Chan, Christakos, & Mattson, 2000). Young adult mice completely lacking calbindin-D_{28k} (cal -/-) have no observable differences in hippocampal structure or number of neurons per section in any region of the hippocampus including CA1, CA3, and dentate granule cells (Gary et al., 2000). As expected, these cal -/- mice show changes in postsynaptic

calcium signaling in the cerebellum and are less active (Airaksinen et al., 1997). Similarly, mice with a regional deficiency of calbindin-D_{28k} in the CA1 region of the hippocampus also exhibited prolonged intracellular calcium levels after stimulation with NMDA agonists or potassium chloride (KCl) (Pasti et al., 1999). These mice with regional deficiency of calbindin-D_{28k} also exhibit slight impairments in acquisition of the radial arm maze and Morris water maze, but not Y-maze performance (Molinari et al., 1996).

These recent findings in which decreases in calbindin-D_{28k} can impair spatial learning may be linked to altered calcium processing. Since calcium is necessary for the release of neurotransmitters from the presynaptic cell (Fink, Meder, Dooley, & Gothert, 2000) and in actions at the postsynaptic level (Schneggenburger & Neher, 2000; Yazejian, Sun, & Grinnell, 2000), it is possible that alterations in calbindin-D_{28K} and subsequently calcium may alter neurotransmitter release important for memory formation (Pasti et al., 1999). Increased intracellular calcium has been observed to impair the acquisition of spatial memory tasks such as the radial arm maze and Morris water maze (Ohnuki & Nomura, 1996; Lanahan, Lyford, Stevenson, Worley, & Barnes, 1997; Garcia, 2001). In addition, increased intracellular calcium associated with depletion of calbindin-D_{28k} leads to decreases in long-term potentiation, the long-lasting increase in the excitability of postsynaptic neurons associated with learning (Molinari et al., 1996). Further, since no changes in nervous system structure have been found in mice with calbindin-D_{28K} deficiency, these observed changes in behavior between cal +/+ and cal -/- mice may be due to alterations in neuronal

signaling and central nervous system communication. Currently, no studies have investigated the effect of calbindin-D_{28k} deletion on neurotransmitter or metabolite levels in the central nervous system.

The present study investigated the effect of calbindin-D_{28k} deletion on learning and memory by exposing male and female null mutant mice to stress conditions. to compare the effect of cal deletion on spatial and non-spatial memory, the spatial radial arm maze and non-spatial object recognition task were utilized. The object recognition task, is a non-reward, non-spatial memory task (Ennaceur & Meliani, 1992) that primarily relies on entorhinal and frontal cortex functioning (Ennaceur et al., 1996; Liu & Bilkey, 2001). The second memory task, the standard radial arm maze, is a spatial, reward-based task that predominantly relies on hippocampal functioning (Li, Matsumoto, & Watanabe, 1999). Based on previously reported and discussed memory impairments in calbindin-D_{28k} deficient models (Molinari et al., 1996), it was hypothesized that calbindin null mutants (cal -/-) would exhibit slightly impaired performance on both memory tasks and that stress would further impair performance due to changes in calcium homeostasis. In addition, sex differences in performance of cal -/- may not be as pronounced as sex differences in cal +/+ controls due to lack of calbindin-D_{28k} during development (Brager et al., 2000). Further, these behavioral changes are expected to be subserved by distinct changes in neurochemical levels in crucial brain regions, such as frontal cortex, hippocampus, and amygdala based on the role of calbindin-D_{28K} in intracellular calcium regulation, the stress response, and learning and memory.

EXPERIMENTAL PROCEDURES

General

Calbindin-D_{28k} null mutant (cal -/-) mice were generated by a targeting approach described in detail elsewhere (Airaksinen et al., 1997). Briefly, calbindin-D_{28k} null mutants were obtained by isolating calbindin-D_{28k} genomic clones from a 129/Sv library. Using a 10.3 kb *Hind*III-*Eco*RI fragment as the targeting vector, a 1.3 kb *Cla*I-*Eco*47III fragment of the calbindin-D_{28k} gene (containing part of the promoter and the first coding exon) was replaced with a 1.6 kb fragment containing the neomycin-resistance (*neo*) gene driven by the PGK promoter and bovine growth hormone polyadenylation signal. The chimeras were crossed to C57BL/6 cal +/+ mice. Heterozygotes (cal +/-) were crossed to obtain cal +/+, cal +/-, and cal -/-.

Thirty-two mice served as subjects. 13 cal+/+ and 19 cal -/- mice (5-6 weeks old), in two cohorts, were transported by car from the University Medical and Dental School of New Jersey and acclimated for one week in Hunter College's animal facility before testing began. Both cohorts in this experiment were exposed to the same conditions, and statistical analysis confirmed no differences between cohorts on any measure. In both cohorts, half the mice from each genotype (total of 6 cal +/+ and 10 cal -/-) were restrained in Broome rodent restrainers (diameter 25.4 mm) for 4 hours daily for 14 days. The 4-hour stress period was split into two 2-hour periods, separated by an hour (in a paradigm similar to that previously described (Zerbib et al., 1990)). Subjects in the chronic stress group were restrained in a separate room in a sound attenuated box during

the 4 hours of stress to diminish effects of any vocalizations or pheromones on non-stressed controls. Stressed subjects were weighed daily following the stress period. Non-stressed controls remained in their home cages and were handled and weighed daily. Subject's weights were analyzed across the stress period with a mixed design, repeated measures ANOVA to assess genotype (cal +/+ vs. cal -/-), stress (control vs. stress), stress day (0 – 14 day), and interaction effects.

Subjects were maintained on a 12:12 reverse light/dark cycle (lights off 9 AM) and tested during the subjective active period starting at 10 AM and 2 PM. Subjects were double housed during acclimation, stress, and open field testing, and singly housed during object recognition testing and radial arm maze testing. Subjects were sacrificed in a separate room during a delay period on the radial arm maze through rapid decapitation. For all behavioral and neurochemical analysis, the primary investigator had a master list of genotype and stress conditions. Others who assisted in gathering behavioral and neurochemical data were blind to condition. For neurochemical analysis, brains were rapidly removed following decapitation, frozen on dry ice, and stored at -60°C for later neurochemical analyses. All subjects were treated under NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

Open Field

Day 1 post-stress: Subjects were placed on a 45 cm. square Plexiglas open field with a 4 x 4 sector grid for 6 minutes. The behaviors recorded across the six

minutes included sector visits, rears, grooms, and fecal boli. A sector visit was defined as a subject bringing at least half of its torso into a sector (the subject could not be in 2 sectors at once). Rearing was defined as a subject raising its upper torso, so that its forelimbs were at least at the position of its head during ambulation. Wall climbs involved the subject raising its torso and placing its front forelimbs on the wall. Between subjects 2 x 2 x 2 ANOVAS were used to assess differences between genotype (cal +/+ vs. cal -/-), stress (non-stress vs. stress), and sex (male vs. female) on all measures of the open field.

Object Recognition

Days 3, 5, 9 post-stress: Object recognition testing consisted of two 5-minute sessions (in the 45 cm square field): a sample trial (T1) and a recognition trial (T2), which were separated by an intertrial interval on subsequent testing days of 10 minutes, 1 hour, and 4 hours. During T1, two identical objects were placed on the field opposite the experimenter. At the beginning of each 5-minute session, the subject was placed on center of the field facing the objects. Exploration of the objects was timed with stopwatches when the subject sniffed, whisked, investigated, or oriented towards the objects from no more than 2 cm away (Beck et al., 1999); (Ennaceur & Aggleton, 1994); (Ennaceur et al., 1997; Ennaceur, Cavoy, Costa, & Delacour, 1989). For each subject, the stopwatch was started when the subject began exploration and the stopwatch was stopped when the subject ceased exploration of the object. After the inter-trial delay, the recognition trial (T2) began; one of the objects was replaced with a novel object. Exploration of the novel and old objects was timed with stopwatches. The total

time spent exploring both the old and new object were recorded and the percentage of time exploring the new object was analyzed (see (Beck et al., 1998) (Ennaceur et al., 1994; Ennaceur & Delacour, 1988; Ennaceur et al., 1989; Ennaceur et al., 1992) for further details). Objects used were determined previously to be equally attractive to mice (unpublished data) and included button covers, small paper binders, sake cups (porcelain and plastic), plastic tea cups, wooden napkin rings, and plastic PVC pieces. The object that served as the old object, and its position (left or right) was counterbalanced across subjects on each trial and different objects were used for each different delay trial. Chance performance in this task leads to an even split in the time spent exploring the objects with an average of 50% exploration of the novel object and 50% exploration of the old object.

2 x 2 x 2 between subjects ANOVAs were used to assess any pre-existing differences in initial exploration time (T1) between the genotypes (cal +/+ and cal -/-), stress conditions (non-stress and stress), and sexes (males and females) at each delay period. For analysis of object recognition, the percent time spent exploring the new object data was transformed by using the arcsine of the square root of the percentage to normalize the distribution (Sokal et al., 1995; Beck et al., 1999; Ennaceur et al., 1988; Ennaceur et al., 1989; Ennaceur et al., 1992; Ennaceur et al., 1994; Ennaceur et al., 1997). This transformed percentage was then tested with between-subjects 2 (genotype: cal +/+, cal -/-) x 2 (stress: non-stress and stress conditions) x 2 (sex: male, female) ANOVAs at each delay period (Beck et al., 1998).

Radial Arm Maze (RAM)

Days 1-12 post-stress: Twenty-four hours prior to testing on the radial arm maze, subjects were food deprived to 90-95% baseline weight, and were maintained around this weight for the duration of testing on the RAM in a paradigm similar to (Luine, Richards, Wu, & Beck, 1998). Subjects were tested twice a day. Subjects were checked daily for dehydration or lethargy, and more food was given if large weight loss was detected. The clear Plexiglas maze sat on a table that was 74.0 cm off the ground. The eight arms of the maze were each 37.3 cm long and 7.7 cm wide with 9.5 cm high walls. Clear Plexiglas tops covered each arm but not the center. The center was octagonal with a 23 cm radius. A number of extra-maze cues were available including a ball of yellow string, a red and white soda can, a green tissue box, two lamps, and black and white checkered wallpaper.

The training/ acquisition trials began in the afternoon after open field testing. A maximum of two trials was run daily. During the 10 acquisition trials, the subject was placed in the center of the eight-arm maze to explore the arms and find the food reward (peanut piece), which was placed at the end of the arm and at 1/3 and 2/3 of the length of the arm. The subject explored the maze for 10 minutes or until all eight arms had been visited. A visit to the arm was recorded if the subject traveled more than halfway down the arm. The total time to complete the task (visit all eight arms), total errors (visiting an arm already visited), errors in the first eight choices, and at what choice the first error occurred were recorded for training/acquisition trials 4 through 10. Performance during acquisition trials

was assessed based on the work of Molinari et al. (Molinari et al., 1996). Percent correct during acquisition was derived from the number correct (8) divided by the total number of choices (errors plus correct responses) and multiplied by 100 based on Molinari et al. (Molinari et al., 1996). The percent correct was analyzed using a mixed design 2 x 2 x 2 x 6 (acquisition trials 4-10) ANOVA to assess differences and interactions between genotype (cal +/+ vs. cal -/-), stress (non-stress vs. stress), sex (male vs. female) and across the repeated factor trials. Regression analysis was run for each genotype between time to complete the maze and total errors to test for possible correlation between activity level and performance.

After the first 10 acquisition trials, 8 testing trials began: subjects were again placed on the maze until they visited all eight arms. During testing trials, the food reward was placed at the end of the arm only. As before, the total time to complete the task, total errors, errors in the first eight choices, and at what choice the first error occurred were recorded. Performance on the 8 testing trials was analyzed using a mixed design 2 x 2 x 2 x 8 (testing trials 1-8) ANOVA to assess differences and interactions between genotype (cal +/+ vs. cal -/-), stress (non-stress vs. stress), sex (male vs. female) and across the repeated factor of 8 trials.

After the 8 testing trials, delay trials began. The subject was taken off the maze between the 4^{th} and 5^{th} choice for an inter-trial delay of 10 minutes, 1 hour, 2 hours, and 3 hours on consecutive trials. After the delay, the subject was placed back on the maze until all the previously unvisited arms were visited. Errors post-delay (visiting an arm previously visited), errors in the first eight choices, and at

what choice the first error occurred were recorded. Delay trial performance was analyzed using a mixed design 2 x 2 x 2 x 4 (delay periods) ANOVA to assess differences and interactions between genotype (cal +/+ vs. cal -/-), stress (non-stress vs. stress), sex (male vs. female) and across the repeated delay periods.

Neurochemical analysis

Following completion of all behavioral testing, subjects were placed on the radial arm maze for four arm choices. Immediately following the four arm choices, each subject was taken into a separate room and sacrificed by rapid decapitation (without anesthesia). The brains were quickly removed and immediately placed on dry ice as previously described (Beck et al., 1998); (Luine et al., 1990; Gogos et al., 1998). Brains were sectioned at 250-300μM at –6 to -8°C in a microtome cryostat. Hippocampal, amygdala, locus coreleus, and pre-frontal cortex brain areas (Figure 3) were micropunched from sections according to the atlas of Franklin and Paxinos (Franklin & Paxinos, 1997) with a 500μM diameter cannula while the slide rested on a microscope stage maintained at –11.5°C, as previously described by (Gogos et al., 1998). Punched samples were placed in 1.4 ml Eppendorf tubes with 60μl of sodium acetate buffer containing α-methyl-dopamine (as internal standard). For frontal cortex samples, micropunched tissue was placed in 75μl of sodium acetate buffer containing α-methyl-dopamine and homoserine (as internal standards). After freeze-thawing and centrifugation, the 60μl supernatant was removed and 2μl of 1 mg/ml ascorbate oxidase solution (Sigma) was added to each sample to minimize the front. Forty microliters were injected in a Waters Associates chromatographic

system consisting of a 717 Plus autosampler, 590 pump, and C-18 reverse-phase 3-μm Nova-pak column (Waters) then an ESA 5011 analytical cell. An ESA Coulochem 5100A electrochemical detector with the screening electrode set at +0.05 V and the detecting electrode at +0.35 V was used. Concentrations of neurotransmitters and metabolites were calculated by reference to standards using peak integration with a computer assisted Waters Millenium system. Sample runs averaged 30 to 40 minutes.

The pellet was then dissolved in 100μl of 0.2 N NaOH for protein determination by the Bradford method (Bradford, 1976). Concentrations are expressed as pg/μg protein. Neurochemical data from each brain region was analyzed using a between subjects 2 x 2 x 2 ANOVA to assess differences and interactions between genotype (cal +/+ vs. cal -/-), stress (non-stress vs. stress), and sex (male vs. female).

For amino acid quantification of frontal cortex samples, the remaining supernatant (15μl) was derivatized with o-phthalaldehyde and ß-mercaptoethanol and injected into a Waters Associates 717 automated refrigerated injection system which maintained samples at –4°C using a 590 pump. The mobile phase (12% methanol and 6% acetonitrile) was pumped through a C-18 reverse phase column (Waters Nova-pak) then an ESA 5011 analytical cell. An ESA 5100A Coulochem detector was set at +0.20 V to oxidize and remove derivation contaminants and +0.40 V to oxidize and detect amino acids. Concentrations and proteins were determined as described above and expressed as ng/μg protein. Runs averaged 40 minutes. Amino acid data was analyzed using a between subjects 2 x 2 x 2

ANOVA to assess differences and interactions between genotype (cal +/+ vs. cal -/-), stress (non-stress vs. stress), and sex (male vs. female).

Statistical Analysis

All data was analyzed using NCSS (Hintze, 1999). Data were checked to ensure a normal distribution using the 3 D'Agostino normality tests for skewness, kurtosis, and omnibus ($p<0.05$) and screened for outliers using a T^2 test based on Mahalanobis distance of each point from the variable means. Fisher's LSD was used for all post-hoc analyses. Probability for rejecting the null hypothesis was set at $p<0.05$. See individual methods sections for specific analyses.

RESULTS

Weight

Stressed subjects of both genotypes failed to gain weight across the stress period compared to non-stressed subjects: $F_{(4, 77)}=14.62$, $p<0.001$, Figure 11. No genotype differences between cal +/+ and cal -/- subjects existed.

Open Field

Cal +/+ mice were more active on the open field. Cal +/+ had more total crossings ($F_{(1,24)}=20.66$, $p<0.001$), more perimeter sector crossings ($F_{(1,24)}=17.01$, $p<0.001$), more inside sector crossings ($F_{(1,24)}=12.76$, $p<0.01$), and more rears ($F_{(1,24)}=23.83$, $p<0.001$) than cal -/-, Table 4. Several sex differences were also found on these measures. Females, of both genotypes, made more total sector crossings ($F_{(1,24)}=4.65$, $p<0.05$) and more inside sector crossings ($F_{(1,24)}=4.67$, $p<0.05$) than males, Table 4. No significant sex differences were found in the perimeter sector crossings, although females tended to make more crossings

Figure 11

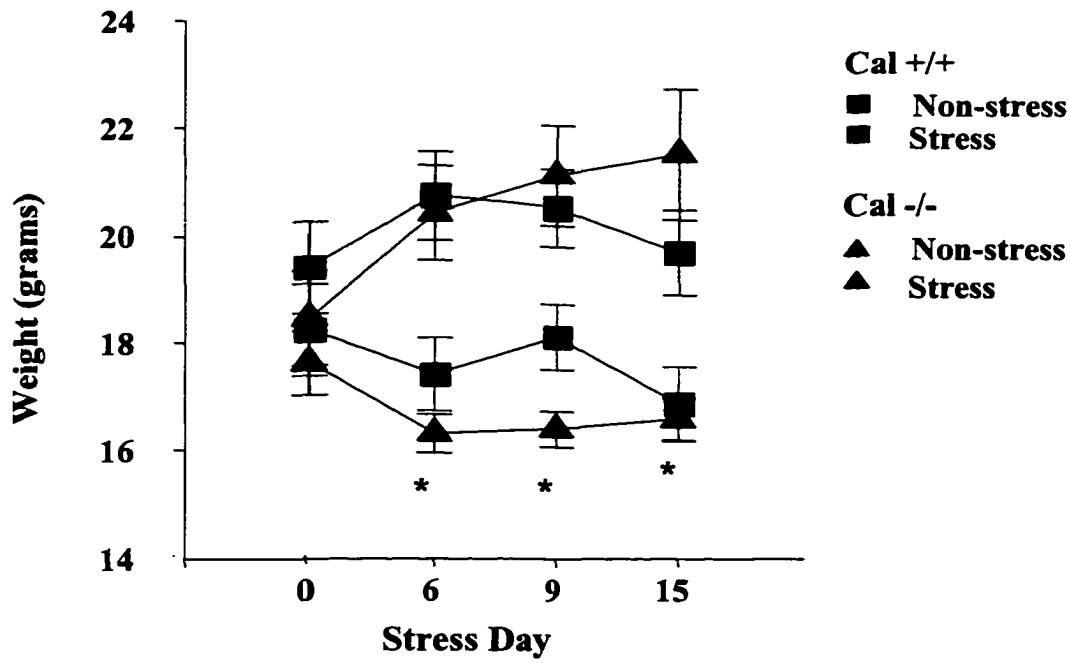


Table 4

	Cal +/+		Cal +/+	
	Male (n=7)	***Female (n=6)***	***Male (n=9)***	***Female (n=10)***
Total Sector Crossings	100.3 ± 9.2*[a]	122.7 ± 14.1*	55.2 ± 5.4[a]	76.0 ± 10.9
Inside Sector Crossings	16.0 ± 4.7*[a]	23.5 ± 3.5*	5.89 ± 1.1[a]	11.7 ± 2.7
Perimeter Sector Crossings	84.3 ± 5.6*	99.2 ± 13.9*	49.3 ± 4.8	64.3 ± 8.6
Ratio Inside/Perimeter	0.18 ± 0.05	0.27 ± 0.05	0.12 ± 0.02	0.16 ± 0.03
Rears	9.3 ± 3.9*	17.8 ± 4.4*	1.0 ± 0.8	1.8 ± 1.2
Grooms	2.3 ± 0.6	1.0 ± 0.4	1.1 ± 0.2	1.4 ± 0.4
Wall Climbs	18.7 ± 3.6	15.5 ± 2.7	6.1 ± 1.8	8.8 ± 2.2
Defecations	2.9 ± 1.0	0.2 ± 0.2	2.9 ± 0.5	2.0 ± 0.3

Activity on the open field in male and female cal +/+ and cal -/- subjects. Asterisk (*) indicates genotype differences in which cal -/- had fewer total crossings, perimeter crossings, inside crossings, and rears than cal +/+ (*$p<0.01$). Symbol ([a]) indicates a signficant sex difference. All females (cal +/+ and cal -/-) had more total crossings and rears ([a] $p<0.05$).

($F_{(1,24)}$=3.20, p=0.086). Stress had little effect on open field activity in either genotype, with the only measure that approached significance being the number of grooms. Stressed subjects had more grooms (mean=1.81 ± 0.3) than non-stressed subjects (mean=1.06 ± 0.3), $F_{(1,24)}$=3.93, p=0.058.

Object Recognition

Analysis of the time spent exploring the 'old' objects during the sample trial (T1), a measure of potential attention or locomotor differences, revealed no significant genotype, stress, or sex differences at any delay period, Figure 12. Specifically, with an inter-trial delay of 10 minutes, there was no difference in exploration time at T1 between genotypes ($F_{(1,25)}$=1.10, p>0.05), stress ($F_{(1,25)}$=0.05, p>0.05), or sex ($F_{(1,25)}$=1.72, p>0.05), Figure 12A. With a 1 hour inter-trial delay, there were no differences in exploration time at T1 between genotypes ($F_{(1,22)}$=0.93, p>0.05), stress ($F_{(1,22)}$=0.01, p>0.05), or sex ($F_{(1,22)}$=0.44, p>0.05), Figure 12B. With a 4 hour inter-trial delay, there were no differences in exploration time at T1 between genotypes ($F_{(1,23)}$=0.69, p>0.05), stress ($F_{(1,23)}$=0.00, p>0.05), or sex ($F_{(1,23)}$=0.65, p>0.05), Figure 12C.

Results from the recognition trial (T2) of the non-spatial task indicate differences between the cal +/+ and cal -/- in both recognition performance and stress effects. On the object recognition with a ten-minute inter-trial delay, all groups spent more than 65% of the time exploring the new object, Figure 13A, confirming that recognition took place since all groups discriminated above chance levels (50%) at this delay. When more cognitive demand was introduced with the longer one-hour inter-trial delay, an interaction between genotype and

stress was significant, $F_{(1, 22)}=8.03$, $p<0.01$, Figure 13B. Fischer's post-hoc ($p<0.05$) indicated that non-stressed cal -/- and stressed cal +/+ subjects spent more time exploring the novel object (70.6% ± 4.0 non-stress cal-/-; 66.1% ± 5.4 stressed cal +/+) than stressed cal -/- subjects (51.9% ± 6.0) or non-stressed cal +/+ control subjects (55.7% ± 2.1), Figure 13B. In contrast to the results with a 1-hour inter-trial delay, no genotype ($F_{(1,22)}=0.07$, $p>0.05$), stress ($F_{(1,22)}=2.57$, $p>0.05$), or interaction ($F_{(1,22)}=0.66$,$p>0.05$) differences existed with a 4-hour inter-trial delay, Figure 13C. At this delay, all groups spent approximately 50% of the time exploring the old and new object, Figure 13C.

Notably, there were no genotype, stress, sex, or interactions in analysis of the total time spent exploring the old and new objects during T2 at any of the delay periods (data not shown). Thus differences in recognition performance are related to how exploration of the old and novel object was partitioned.

Briefly, these results indicate that stress enhanced non-spatial memory performance in the cal +/+ subjects since stressed subjects spent significantly more time exploring the novel object at the one-hour delay. Baseline performance of non-stressed subjects of both genotypes indicated that the non-stress cal -/- subjects performed better than the non-stressed cal +/+ animals. However, stress severely impaired performance of the cal -/- subjects. Thus, it appears that stress enhanced performance on this memory task in unaltered, cal +/+ subjects but debilitated cal -/-.

Figure 12

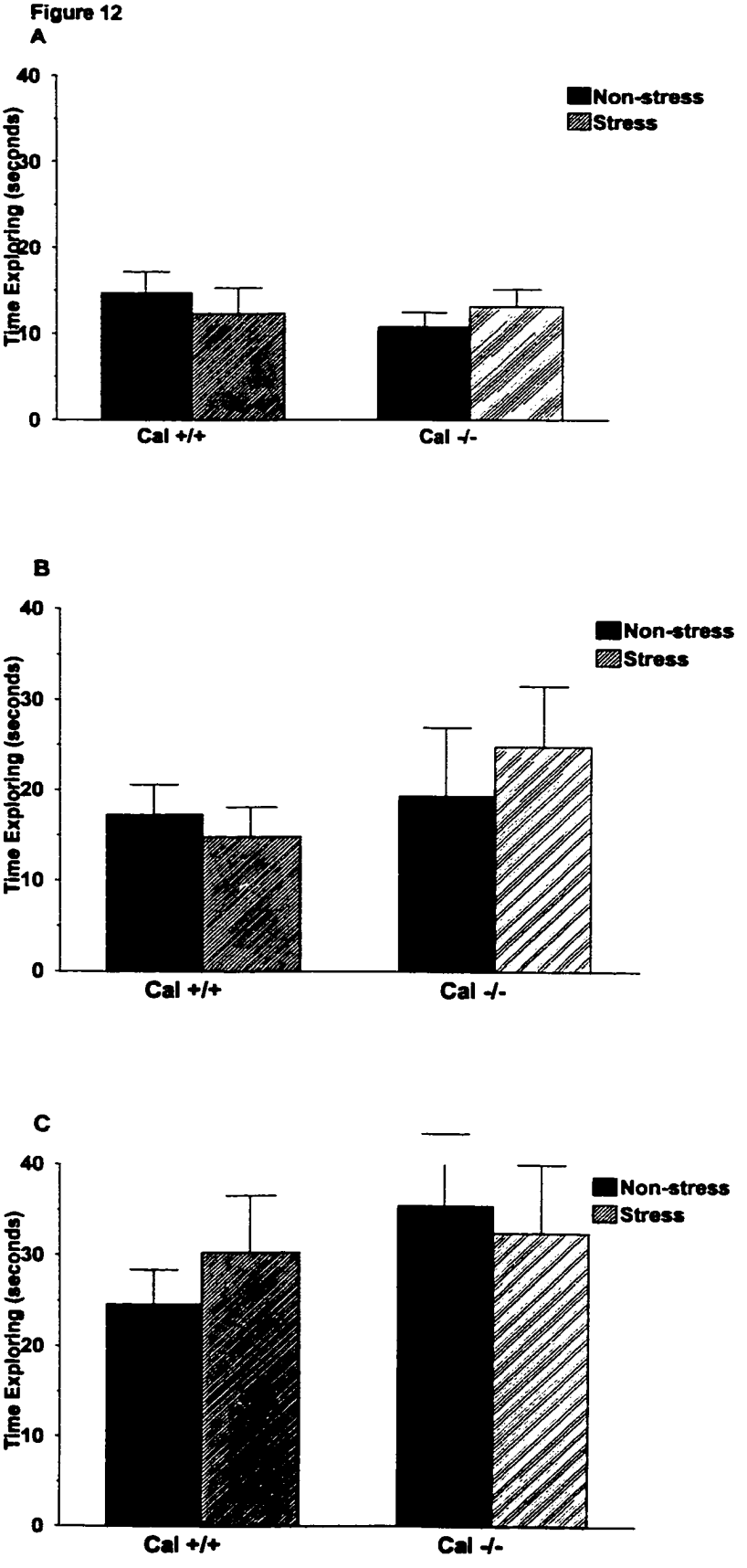
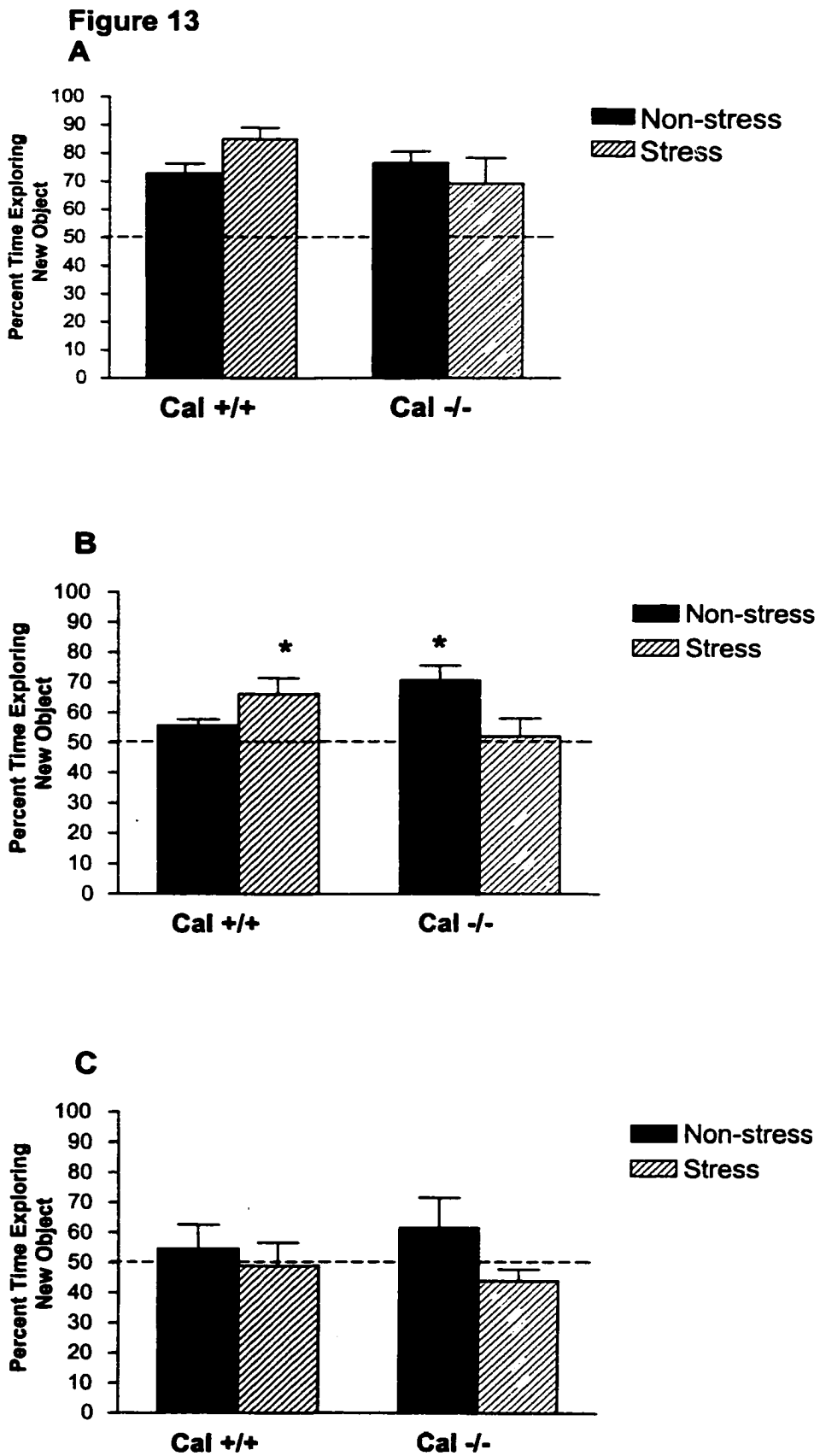


Figure 13



Radial Arm Maze (RAM)

Training/ Acquisition Trials (RAM)

The percent correct during acquisition was calculated to compare results to the findings of Molinari et al. (Molinari et al., 1996). During acquisition, there were no significant main effects of genotype, stress, or sex. However, a genotype by sex interaction ($F_{(1,24)}$=4.51, $p<0.05$) and subsequent post-hoc analysis with Fisher's LSD ($p<0.05$) revealed a sex difference in the cal +/+ genotype but not the cal -/- genotype. There was no significant difference between cal -/- males (69.7% ± 2.1) and females (67.3%± 2.1); however, cal +/+ females had significantly lower percent correct (61.3% ± 2.6) than cal +/+ males (73.7% ± 2.4), data not shown. Null mutation of calbindin-D_{28k} therefore appears to attenuate the sex difference, since male and female null mutants performed the same, whereas significant sex differences were found in cal +/+ controls.

Testing Trials (RAM)

After acquisition trials, cal -/- took significantly longer to complete the radial arm maze task than cal +/+ ($F_{(1,231)}$=7.53, $p<0.05$), Figure 14A; however, there was no correlation between time to complete the maze and total errors for either cal +/+ (r^2=0.08, $p>0.05$) or cal -/- subjects (r^2=0.006, $p>0.05$), Figure 14B. Since there is a significant difference in time to complete the maze, this variable was used as a covariate in the analysis of performance on the radial arm maze trials. Time to complete the maze was a significant covariate ($F_{(1,221)}$=28.11,$p<0.05$). Using the corrected model with time to complete the maze as a covariate, there was no significant genotype difference in radial arm

maze performance, similar to acquisition trials. However, a sex difference in performance was still present with males continuing to exhibit better performance. A significant sex effect, $F_{(1,23)}=13.41$, $p<0.01$, revealed females made more total errors (4.37 ± 0.3) compared to males (2.59 ± 0.3), Figure 15. The sex difference is consistent with findings in rats in which females do not perform as well as males in spatial memory tasks (Luine et al., 1994b; Luine et al., 1998; Williams, Barnett, & Meck, 1990). There was no sex difference in time to complete the task, $F_{(1,231)}=1.24$, $p=0.26$. In addition, there were no differences in the number correct in the first eight choices, (genotype: $F_{(1,23)}=0.01$, $p>0.05$, stress: $F_{(1,23)}=0.19$, $p>0.05$, sex: $F_{(1,23)}=1.98$, $p>0.05$) Figure 16.

Delay Trials (RAM)

As more cognitive demand was introduced into the radial arm maze task by placing delays between the fourth and fifth arm choice, stress effects emerged. Stress significantly impaired performance of all subjects, regardless of genotype. Stressed subjects had more total errors, $F_{(1,24)}=5.79$, $p<0.05$. This result is in contrast to the object recognition results in which the genotypes responded differently to stress, Figure 17.

With inter-trial delays of increasing lengths (10 minutes, 1 hour, 2 hours, and 3 hours) between the fourth and fifth choices, all subjects made more errors in the first eight choices as the delays increased from 10 minutes to 3 hours, confirming increased cognitive demand (Delay Period Effect: $F_{(3,67)} = 2.82$, $p<0.05$), data not shown.

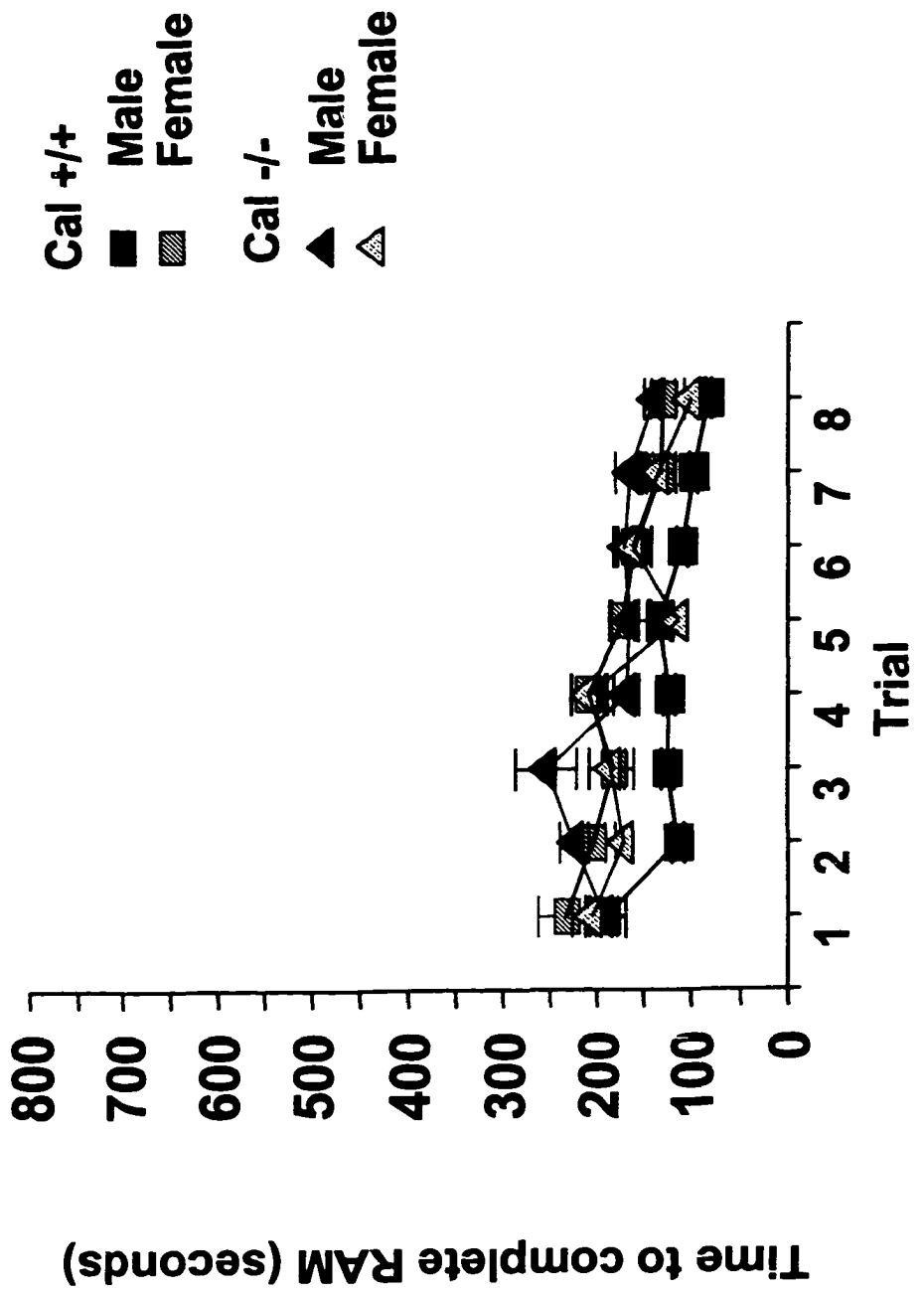


Figure 14A

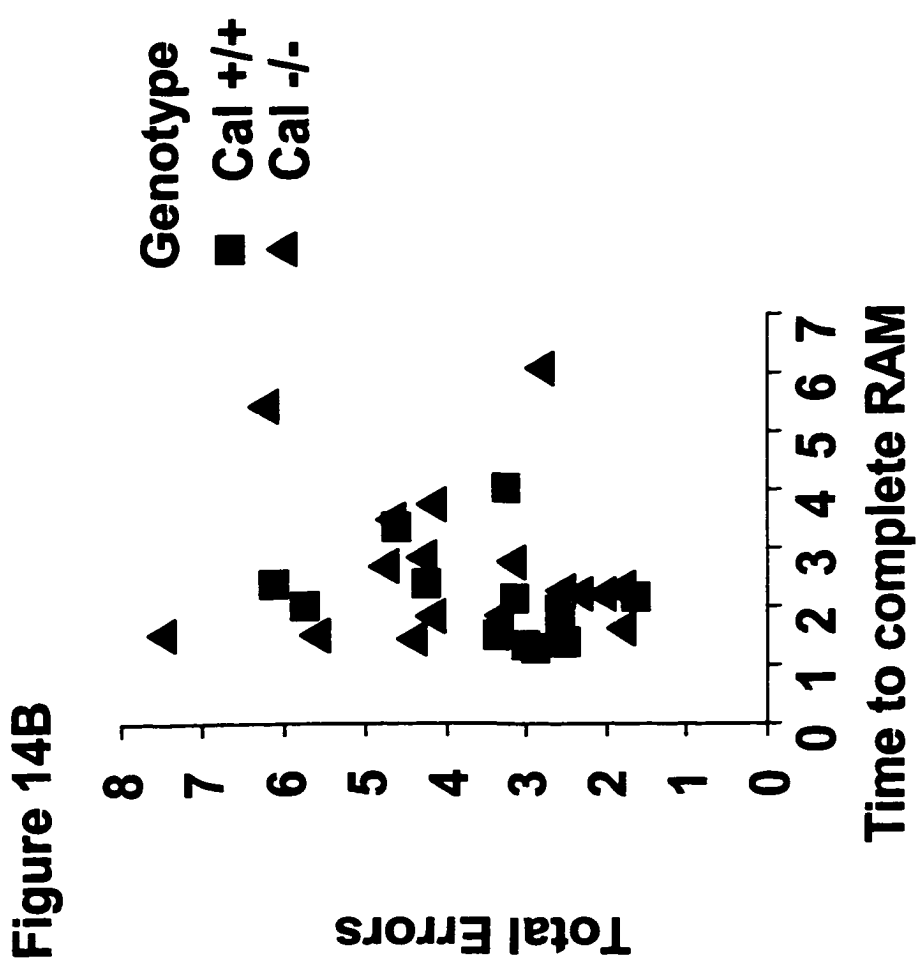


Figure 14B

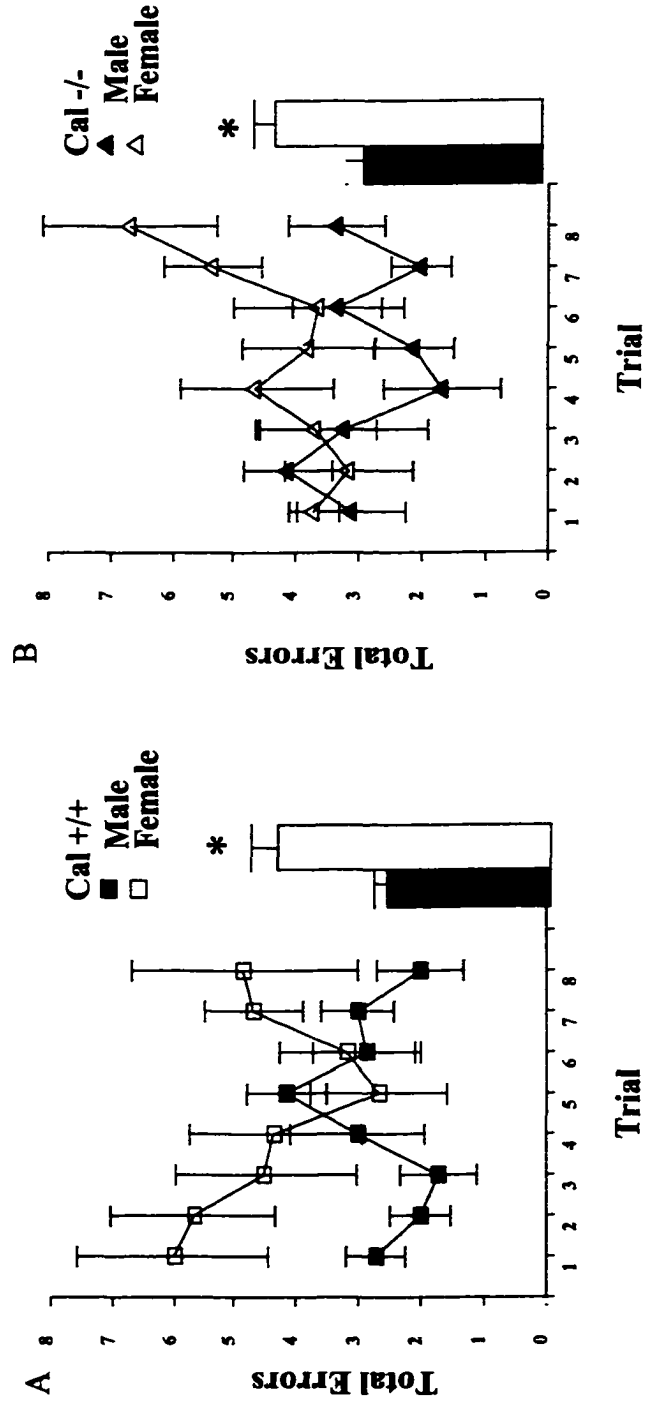


Figure 15

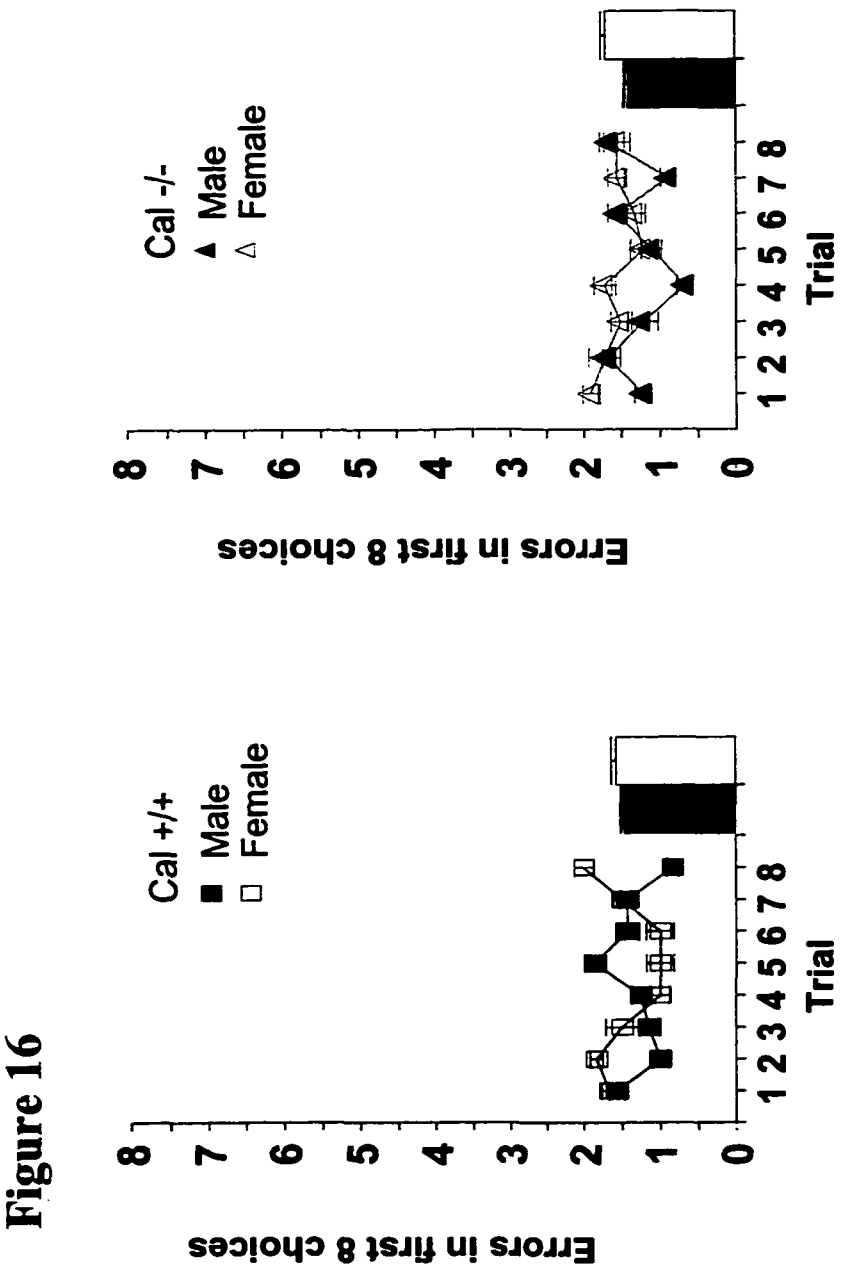
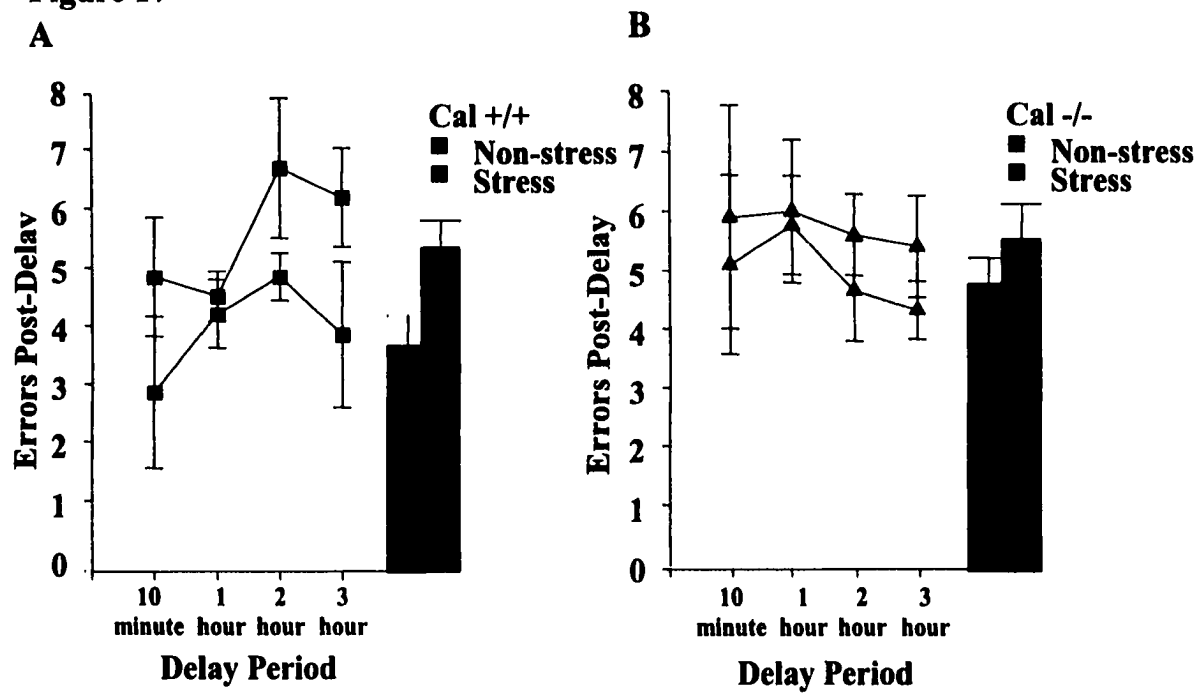


Figure 16

Figure 17



Neurochemical Analysis

Immediately after visiting 4 arms of the radial arm maze, mice were sacrificed and brain areas implicated in memory were neurochemically analyzed. In the hippocampus, a significant genotype x stress effect for MHPG ($F_{(1,22)}$=4.25, $p<0.05$), norepinephrine (NE) ($F_{(1,22)}$=6.65, $p<0.02$), and 5HIAA ($F_{(1,22)}$=4.25, $p=0.05$) was observed, Table 5B. Overall stressed cal +/+ subjects showed a significant increase in NE and its metabolite, MHPG, as well as a significant increase in the 5-HT metabolite, 5HIAA. Fisher post-hoc analysis ($p<0.05$) revealed stressed cal +/+ subjects have significantly more MHPG than stressed cal -/- subjects. Stressed cal +/+ subjects also have significantly more NE and 5HIAA than any other group as revealed with Fisher's post-hoc test ($p<0.05$). Further, there was a significant sex difference in 5-HT levels ($F_{(1,23)}$=4.66, $p<0.05$) in which females had lower levels than males. There was a slight difference between males and females in turnover of 5-HT into 5HIAA as measured by the ratio 5HIAA/5-HT ($F_{(1,23)}$=3.20, $p=0.087$) in which females had a slightly higher ratio (1.5 +/- 0.097) than males (1.19 +/- 0.099).

In the amygdala, significant genotype effects on monoamine and metabolite levels reveal that cal -/- subjects had lower overall levels of MHPG ($F_{(1,22)}$=13.27. $p<0.01$), NE ($F_{(1,22)}$=22.34, $p<0.001$), and 5HIAA ($F_{(1,23)}$=7.02, $p=0.01$, Table 5A. A significant sex effect ($F_{(1,22)}$=7.76, $p<0.02$) revealed that levels of NE were significantly lower in females in the amygdala (data not shown). No significant differences were observed in 5-HT levels.

Table 5

A

Amygdala	NE	MHPG	5HT	5HIAA
Cal +/+ Non-stress	5.43 ± 0.56*	2.69 ± 0.26*	7.34 ± 1.59	9.99 ± 1.38*
Cal +/+ Stress	6.38 ± 0.56*	2.68 ± 0.26*	8.29 ± 1.57	6.40 ± 1.38*
Cal -/- Non-stress	3.14 ± 0.49	1.71 ± 0.22	6.93 ± 1.28	4.66 ± 1.12
Cal -/- Stress	3.84 ± 0.43	1.94 ± 0.20	8.51 ± 1.28	.08 ± 1.07

B

Hippocampus	NE	MHPG	5HT	5HIAA
Cal +/+ Non-stress	4.56 ± 1.03	2.59 ± 0.51	4.51 ± 0.66	6.42 ± 0.96*
Cal +/+ Stress	8.19 ± 1.13*	3.63 ± 0.56	6.06 ± 0.72	8.08 ± 1.17*
Cal -/- Non-stress	5.63 ± 0.89	2.85 ± 0.44	4.41 ± 0.57	5.50 ± 0.78
Cal -/- Stress	4.89 ± 0.80	2.31 ± 0.39	3.75 ± 0.53	5.23 ± 0.74

C

Frontal Cortex	NE	MHPG	5HT	5HIAA
Cal +/+ Non-stress	4.97 ± 0.73*	Not detected	Not detected	3.75 ± 1.03
Cal +/+ Stress	6.68 ± 0.90*	Not detected	Not detected	4.75 ± 1.15
Cal -/- Non-stress	4.25 ± 0.60	Not detected	Not detected	4.61 ± 0.77
Cal -/- Stress	4.66 ± 0.60	Not detected	Not detected	4.72 ± 0.77

D

Locus Coreleus	NE	MHPG	5HT	5HIAA
Cal +/+ Non-stress	8.02 ± 1.40*	2.91 ± 0.97	3.24 ± 0.62	7.63 ± 0.81
Cal +/+ Stress	9.29 ± 1.40*	2.39 ± 0.97	1.73 ± 0.61	8.43 ± 0.80
Cal -/- Non-stress	5.92 ± 1.45	2.01 ± 0.79	1.27 ± 0.50	8.24 ± 0.66
Cal -/- Stress	6.12 ± 1.08	1.47 ± 0.75	1.77 ± 0.47	7.49 ± 0.63

Changes in monoamine levels in the amygdala (A), hippocampus (B), frontal cortex (C), and locus coeruleus (D) of stressed and non-stressed cal +/+ and cal -/- subjects. Asterisk (*) indicates a significant genotype difference in NE, MHPG, and 5-HT in the amygdala (A), in 5HIAA in the hippocampus (B), and in NE in the frontal cortex (C) and locus coreleus (D). Symbol ($^{+}$) indicates a significant genotype by stress interaction in which stressed cal +/+ subjects (n=6) had significant increases in NE ($^{+}p<0.05$) whereas stressed cal -/- (n=10) did not have a comparable increase in NE. Memory impairments may be due to inability of the cal -/- subjects to increase NE in response to stress.

In the frontal cortex, dopamine (DA) and 5-HT were not detected in most samples thereby precluding statistical analysis. However, NE was detected in all samples and was reduced in cal -/- subjects ($F_{(1,23)}$=4.64, $p<0.05$), and increased overall in both stressed cal +/+ and cal -/- subjects, ($F_{(1,23)}$=8.89, $p<0.01$), Table 5C. Similar significant decreases in NE in the locus coeruleus were found in cal -/- subjects ($F_{(1,23)}$=4.42, $p<0.05$), Table 5D.

There were no significant main effects or interactions in analysis of genotype, stress or sex for glutamate or GABA levels in the frontal cortex or locus coeruleus (data not shown).

DISCUSSION

Results from this study confirm a number of the original hypotheses. First, null mutation of calbindin-D_{28k} diminished that ability of the central nervous system to counteract stress insults. Stressed cal -/- exhibited poor performance on both the object recognition and radial arm maze tasks. In addition, cal -/- had alterations in the stress response in relation to both performance on the object recognition task and to changes in neurotransmitter levels compared to cal +/+. Contrary to expectation, cal -/- subjects were not impaired on the non-spatial object recognition task, and in fact, showed enhanced performance compared to cal +/+ controls. In contrast, no differences between cal +/+ and cal -/- subjects were found on spatial radial arm maze performance. As predicted, observed behavioral differences were subserved by distinct variation in neurochemical levels in several brain areas related to learning, memory, and stress reactivity. Further, as expected sex differences were not as pronounced in cal -/- subjects as

in cal +/+ subjects although these results varied depending on the nature of the task. These results suggest that there is a range of calbindin-D_{28K} expression in the brain that is optimal for locomotor activity, spatial and non-spatial learning and memory, and resistance to chronic environmental stress and that these behaviors are influenced by alteration in neurotransmitters which are affected by calbindin-D_{28k} levels.

Genotype Differences

Cal -/- performed better than cal +/+ controls on the object recognition task. In this non-spatial memory task, non-stressed cal -/- spent significantly more time exploring the novel object compared to non-stressed cal +/+ controls when a one-hour inter-trial delay was placed between the sample (T1) and recognition trials (T2). As expected due to the abundance of calbindin-D_{28k} in the cerebellum of wild-type animals, cal -/- were less active on all measures of the open field. While the potential exists that differences in activity may confound interpretation of behavior performance, our results have found that the cal -/- who had *decreased* activity on the open field had *enhanced* performance on the object recognition task. Further, there is no evidence that a decrease in activity in cal -/- affected exploration of objects in the object recognition task as there was neither a difference in initial exploration time in the sample trial nor a difference in total exploration time during the recognition trial. In addition, potential differences in activity did not affect interpretation of radial arm maze data as the correlation between time to complete the task and performance was very low for both cal +/+

and cal -/- and this possible influence was controlled statistically by incorporating activity as a covariate in the analysis.

In contrast to the object recognition performance and as expected, cal -/- did not perform better than cal +/+ on the radial arm maze. These findings are similar to those of Molinari et al. (Molinari et al., 1996) who reported only a slight impairment in acquisition of the radial arm maze in calbindin-D_{28k} deficient mice. As discussed by Molinari et al. (1996), calbindin-D_{28k} deficient mice did not show general learning differences from controls but had possible selective impairment in acquisition of spatial processing. Impairments in their study on the radial arm maze were found in only 2 out of 10 acquisition trials (Molinari et al., 1996). Minor impairment was also found in a select 2 out of 16 trials of the spatial Morris water maze (Molinari et al., 1996). Our testing extended beyond acquisition, and while our results failed to pick up this slight impairment, further testing revealed no additional differences between cal +/+ and cal -/-. Minor differences in procedures between the studies include differences in the amount of calbindin-D_{28K} which was expressed in the calbindin-D_{28k} null mutant mutants. The mice used in the Molinari et al. (1996) study were created with an antisense gene insertion and were not a complete null mutant of the calbindin-D_{28K} protein. In addition, subjects in the Molinari et al. (1996) study were water deprived and reinforced with a sugar solution while in our study the mice were food deprived and reinforced with peanuts. These small differences in calbindin-D_{28k} expression and reinforcement procedures may have resulted in the minute differences in the results of the two studies.

The main genotype difference in this study was found in object recognition performance in which cal +/+ had inferior performance compared to cal -/-. However, no significant differences were found in radial arm maze performance. These results are consistent with dichotomous performance on spatial versus non-spatial memory tasks previously reported in calbindin-D_{28k} deficient mice (Molinari et al., 1996). Since each of these tasks relies on different brain regions and calbindin-D_{28k} expression varies from one brain region to another, it is not surprising that there are differences in the effect of the deletion on these tasks. Object recognition is a non-spatial, non-reward task that is dependent, in part, on attentional aspects of the frontal cortex (Ennaceur et al., 1994; Ennaceur et al., 1997) and the entorhinal cortex (Ennaceur et al., 1996). Conversely, the radial arm maze requires spatial cues and is dependent on hippocampal learning (see Robbins, McAlonan, Muir, & Everitt, 1997 for review). Other differences between the tasks include that the radial arm maze requires extensive training, food deprivation, and reward based learning whereas the object recognition task is non-reward based, does not require food or water restriction, and can be assessed without prior training of the animals. In summary, our results indicate that deletion of calbindin-D_{28k} did not enhance hippocampal based learning whereas this deletion did enhance performance in frontal or entorhinal cortex based learning.

Stress Effects

Stressed subjects of both genotypes failed to gain weight across the stress period, thus confirming that the 14-days of restraint was an effective stressor.

These results corroborate previous results that stress can lead to weight loss (e.g. (Willner et al., 1996; Magarinos et al., 1995; Krugers et al., 1996; Beck et al., 1999). In learning and memory tasks, stress had different effects on cal -/- and cal +/+ subjects depending on the task itself. Stress impaired memory performance of cal -/- on both the object recognition and radial arm maze tasks. In cal +/+ subjects, stress enhanced object recognition and impaired radial arm maze performance. These differences in the effects of stress may be due to a number of distinguishing features of each task. Differences in stress effects on task performance suggest that stress enhanced frontal cortex dependent attention and learning but impaired hippocampal learning in cal +/+ subjects. In contrast, stress impaired performance on both the radial arm maze and object recognition tasks in cal -/- mice. Cal -/- subjects may have altered intracellular calcium levels and thus altered release of and sensitivity to excitatory neurotransmitters necessary for learning and memory (Brager et al., 2000). However, when confronted with stress, subjects with null mutation of calbindin-D_{28K} may lack the ability to mediate calcium influx and thus have less resistance to the damaging effects of increased excitatory neurotransmitter release associated with chronic stress. Recent studies investigating the excitatory pathways have found that kainic acid, a glutamate agonist, does not appear to affect vulnerability of the CA1 area of the hippocampus in calbindin-D_{28K} -/- subjects (Gary et al., 2000), but no studies have investigated similar vulnerability in a calbindin-D_{28k} -/- system or vulnerability under stress conditions. As suggested by our data discussed below in the neurochemistry section and previous research (Klapstein et al., 1998), the

integrity of intracellular mechanisms including action potential properties may be altered in cal-/- subjects. These internal mechanisms may not be resistant to stress induced changes, a finding which is supported by our neurochemical results showing differential alterations in neurotransmitter levels and metabolism following stress in cal -/- compared to cal +/+ subjects.

A specific alteration in calbindin-D_{28k} may lead to changes in calcium levels, thus, altering properties of neurons in brain regions responsive to stress and important in learning and memory (Klapstein et al., 1998). Recent evidence has, in fact, shown alterations in calcium levels in neurons of cal -/- subjects (Pasti et al., 1999; Klapstein et al., 1998) which may alter both neuronal function and structure. Calcium related functions in neurons are well known and alterations in Ca^{2+} can significantly alter several aspects of neuronal functioning. Agonist induced increases in calcium have been shown to inactivate NMDA receptor-channels in hippocampal and cerebellar neurons (Legendre, Rosenmund, & Westbrook, 1993; Medina, Filippova, Bakhramov, & Bregestovski, 1996; Medina et al., 1995; Rosenmund, Feltz, & Westbrook, 1995). This down regulation of NMDA receptor activity may partially account for the enhancement in learning of cal -/- since the calbindin-D_{28K} null mutant system may be adapted to respond to circulating levels of glutamate. However, when overloaded by chronic stress, down regulation of these receptors may not be sufficient to counteract the overstimulating effects of stress. Recent evidence from calbindin-D_{28K} deficient mice indicates that there is in fact a decrease in NMDA receptor mediated responses while non-NMDA responses are increased (Jouvenceau et al., 1999).

This decrease could not be ascribed to changes in NMDA channel properties of the calbindin-D_{28K} null mutant subjects and therefore probably reflect altered neuronal function as well as resistance to stress-related changes (Klapstein et al., 1998).

Neurons from calbindin-D_{28K} null mutants (cal -/-) have been shown to have increased depolarization induced calcium rises and prolonged elevation of the calcium signal (Airaksinen et al., 1997; Pasti et al., 1999). Specifically, changes in glutamate release may be altered in calbindin-D_{28K} null mutants (cal -/-) and may be a direct result of lack of calbindin-D_{28K} in the cell itself. Altered glutamate transmission may increase vulnerability of the cell to stress. Alternatively, calbindin-D_{28K} null mutants (cal -/-) may upregulate other calcium binding proteins. Although no upregulation of calretinin, parvalbumin, calbindin-D_{9K}, calmodulin, or S100β was observed compared to cal +/+ subjects, the possibility that another unexamined or unknown calcium binding protein is upregulated cannot be dismissed (Airaksinen et al., 1997).

Sex Differences

Consistent with previous reports, males were less active on the open field and had fewer errors on the radial arm maze compared to females (Luine et al., 1994b; Beck et al., 1998; Williams et al., 1990). The sex differences on the radial arm maze were somewhat inconsistent in cal +/+ and cal -/-. Specifically, there was no significant sex difference in performance on acquisition trials of the radial arm maze in cal -/- subjects, suggesting that calbindin-D_{28K} may be affecting sexual differentiation of brain regions involved in spatial memory acquisition.

This is consistent with recent findings that males and females express different levels of calbindin-D_{28k} in several brain regions (Brager et al., 2000; Sickel et al., 2000; Brager et al., 2000; Taylor et al., 1999; Watson et al., 1998; Lephart et al., 1997; Lephart, 1996). Lack of calbindin during development may therefore have slightly attenuated sexual differentiation of brain regions. Further studies on the specific effects of calbindin-D_{28k} deletion on sexual differentiation may elucidate the basis for the decrease in sexual dimorphism of cal -/-.

As discussed above, changes in glutamatergic signaling caused by calbindin-D_{28k} absence may be affected by and may affect neurochemical signaling (Jouvenceau et al., 1999). Differences in behavior may be partially attributed to observed changes in monoamine and metabolites in several brain regions in cal -/- compared to cal +/+. As predicted and consistent with behavioral differences, significant differences between cal +/+ and cal -/- subjects emerged for several monoamines and their metabolites in several brain regions. Most notably, norepinephrine (NE) was lower in cal -/- subjects in three brain regions investigated: the frontal cortex, amygdala, and locus coreleus. In addition, in the amygdala, levels of the NE metabolite, MHPG, were lower in cal -/- subjects. Previous studies have investigated the potential relationship between NE and learning and found similar results. Specifically, Landers and Sullivan (1999) showed that injections of NE could block acquisition of conditioned responses. Similarly, in our study, the cal +/+ subjects, which exhibited higher levels of NE, showed worse performance on the object recognition task compared to cal -/- subjects. The object recognition task has previously been shown to rely, in part,

on frontal cortex function (Ennaceur et al., 1997). Thus differences in NE in the locus coreleus and the frontal cortex upon which it projects may affect attentional components necessary for memory on a non-spatial task.

Notably, reactivity of monoamine systems to stress in cal -/- subjects was *not* similar to reactivity in cal +/+ subjects. Specifically, in the hippocampus, several monoamines were elevated in stressed cal +/+ subjects but did not show similar increased in stressed cal -/- subjects. Specifically, levels of NE and its metabolite, MHPG, were higher in stressed cal +/+ subjects than any other group. Results from these cal +/+ subjects confirms previous findings that a variety of chronic stressors increase NE release (for review see (Shanks et al., 1991) (Shanks et al., 1994) (Beck et al., 1998) (Weiss & Simson, 1988)). As suggested by Zalcman, et al. (1993) the increase in NE in response to chronic stress enables the animal to deal with environmental demands. Our results suggest that the increase in NE in response to chronic stress permitted the cal +/+ subjects to perform effectively on the non-spatial object recognition task. However, stressed cal -/- subjects did not exhibit increased NE in response to chronic restraint and displayed poor performance on both memory tasks. This inability to increase NE may have led to diminished ability to cope with the environmental challenges and memory demands of both the object recognition and radial arm maze tasks. Further investigation into the mechanism by which stress increases NE release and its specific role in memory function may provide an understanding of the role of calbindin-D_{28K} or calcium mobilization in this response.

One of the cellular mechanisms that may influence neurotransmitter levels is calcium mobilization. Previous studies have found that decreased calcium mobilization impairs avoidance performance and spatial radial arm maze performance (Ohnuki et al., 1996). It is therefore possible that any changes in calcium mobilization in cal -/- may be influence memory processes (Jouvenceau et al., 1999; Klapstein et al., 1998). Further, recent studies with cal -/- have shown alterations in action potential firing (Klapstein et al., 1998) and long-term potentiation, a mechanism considered important in memory formation (Molinari et al., 1996). Our results indicate that there are significant differences in learning and memory processing and in the stress effects on learning in cal -/- subjects which may be influenced by changes in calcium mobilization, action potential firing, as well as long term potentiation.

Several studies have reported highly transient changes in monoamine concentrations depending on time post-stress when the monoamines were measured (Beck et al., 1998). Dopamine (DA) and serotonin (5-HT) appear be more susceptible to transient changes (Finlay et al., 1995; Weiss, Bailey, Pohorecky, Korzeniowski, & Grillione, 1980; Cabib et al., 1991). Therefore, it is not surprising that few changes in either DA or 5-HT or their metabolites were detected in this study since brains were extracted almost 2 weeks after the application of stress. However, our results do not preclude changes in these monoamines at earlier intervals following stress or in other brain areas.

Conclusion

In conclusion, our data suggest that calbindin-D_{28k} is involved in the stress response, several pathways underlying this response, as well as behavioral functioning. Therefore, an optimal amount of calbindin-D_{28k} may be necessary for different types of learning, memory, locomotion, and response to stress. This optimal level may not be the same for all of these processes. Calbindin-D_{28k} deletion enhanced performance on the non-spatial memory task and did not affect spatial memory performance compared to wild-type controls. However, chronic stress appears to compromise the ability of the calbindin-D_{28k} null mutant system to compensate as mice lacking calbindin-D_{28k} had impaired performance on both memory tasks following stress and lacked neurochemical compensation to the environmental stress. Inability to compensate for stress in calbindin-D_{28k} -/- subjects may be due to differences in intracellular calcium signaling, neurotransmitter release and use, and action potential characteristics. Our neurochemical evidence suggests significant disruption of the noradrenergic system which may underlie differences in stress reactivity and learning and memory between cal +/+ and cal -/-. Further in vivo and in vitro studies are clearly necessary to understand these results and clarify which neural mechanisms underlie genetic and environmental effects. This study illustrates the usefulness of studying the stress response in null mutant models. Taken together, these results suggest that alteration of a system involved in central nervous system processes may not immediately result in impairments but may compromise the ability of the organism to deal effectively with environmental demands.

Chapter 4

Catechol-*O*-Methyltransferase: Role of catecholamines in stress response

INTRODUCTION

Chronic stress has been found to alter behavioral, endocrine, and neurochemical properties through increased arousal of the hypothalamic-pituitary adrenal axis (HPA) (McEwen et al., 1993). These alterations may be an attempt by the organism to return the system to homeostasis after environmental challenge (Cannon, 1935). Important in the return of the system to homeostasis is the ability of the system to counteract the effects of heightened HPA axis arousal. One aspect of this is the ability to metabolize neurotransmitters that have been elevated in the presence of the stressor.

One of the mechanisms for metabolizing neurotransmitters, specifically the catecholamines, is the enzyme catechol-*o*-methyltransferase (COMT). This enzyme breaks down dopamine, norepinephrine, and epinephrine through *o*-methylation of a methyl group to a hydroxyl group (Grossman, Emanuel, & Budarf, 1992; Axelrod & Tomchick, 1958). While COMT is present in many brain regions, it is primarily responsible for catabolism in the frontal cortex (Karoum, Chrapusta, & Egan, 1994). This is due to low expression of the complimentary enzyme, monoamine oxidase (MAO), in the frontal regions of the brain (Karoum et al., 1994). MAO is an enzyme which also metabolizes these neurotransmitters (in addition to serotonin) but is found primarily in the hippocampal and caudal brain regions (Karoum et al., 1994). Pharmacological

evidence indicates that COMT inhibitors fail to affect dopamine levels in caudal brain regions such as the striatum unless paired additionally with a dopamine carrier system or MAO-A inhibitor (Kaakkola & Wurtman, 1993). In addition, COMT is absent from the dopaminergic terminals and is thought to be involved in catabolism of only extraneuronal dopamine (Napolitano & Cesura, 1995).

COMT may be an important enzyme in returning catecholamine levels to pre-stress levels following disruption of the homeostatic state. Previous reports have found alterations in catecholamine levels in frontal cortex regions as well as alterations in frontal cortex dependent behaviors such as the non-spatial object recognition task e.g. (Beck et al., 1999; Cabib et al., 1988). Specifically, Finlay et al., (1995) have found increases in both dopamine and norepinephrine release in response to chronic stress in the prefrontal cortex. Further, pharmacological priming of dopaminergic and noradrenergic systems with agonists and antagonists of these neurotransmitters suggest sensitization of these systems as a result of stress (Cabib et al., 1997; Badiani et al., 1992).

Different levels of genetic and phenotypic expression of COMT may affect how well this enzyme is able to counteract the stress effects. There are two allelic forms of the COMT gene which can combine into three possible genotypes. The difference between the two alleles of COMT are due to a transition of guanine to adenine at codon 158, which results in a switch from the valine-108 (val-108, high activity allele) to a methionine-108 (met-108, low activity allele). These two alleles can result in homozygosity of val-108 (2 copies of val-108), homozygosity of the met-108 (2 copies of met), or heterozygosity

(one copy of each allele). Homozygosity for the low activity met-108 allele leads to a 3-4 fold reduction in enzymatic activity compared to homozygosity for the high activity val-108 allele. Heterozygosity results in intermediate activity (Lachman et al., 1997). Reduced levels of COMT activity, through the met-108 allele or known as met-met genotype, result in increased dopamine and norepinephrine since there is less COMT available for catabolizing the neurotransmitter.

These existing genetic differences have implications for stress reactivity. A prevailing premise in both stress and genetic research is the vulnerability hypothesis which contends that stress can interact with a genetic predisposition to trigger illness onset (Dohrenwend et al., 1981a). In addition to potential influences on stress effects, COMT has been implicated for its role in genetic basis of several disorders. The recessive low-activity COMT (met-108) allele has been shown to be a risk factor for both schizophrenia and obsessive-compulsive disorder (OCD) (Karayiorgou et al., 1995; Karayiorgou et al., 1997). In addition, evidence indicates bipolar subjects have lower allelic expression of COMT (met-met genotype) than non-affected controls (Li et al., 1997; Cohn, 1997; Karege, Bovier, Gaillard, & Tissot, 1987).

Insight into the effects of stress in subjects with different levels of COMT expression can provide information about the role of COMT in the stress response. In addition, study of COMT null mutants can provide understanding of the complex interaction between genetic and environmental factors. Recently, the development and analysis of the COMT null mutant (COMT -/-) mouse has

confirmed a number of hypotheses concerning the role of COMT (Gogos et al., 1998). Male COMT -/- mice show levels of dopamine consistent with the higher levels seen in psychiatric patients, a two to three fold increase in dopamine in the frontal cortex (Lachman et al., 1996). Interestingly, a similar increase was not observed in the female COMT -/- (presumably due to a compensation as yet undescribed). This finding is consistent with observations in humans that males with low activity alleles show more OCD symptoms than females (Karayiorgou et al., 1999). As expected, no changes of dopamine levels in the hypothalamus or striatum for males or females were observed (Gogos et al., 1998). This observation confirms that the mechanism of *o*-methylation in breaking down dopamine occurs predominately in the frontal cortex and is not primarily responsible for catabolism in the striatum (Karoum et al., 1994). Thus, behavioral differences seen in COMT -/- mice may be due to differences in frontal cortex dopamine levels. Behavioral differences appear to be sex dependent, consistent with the neurotransmitter differences. In a test of anxiety using the dark/light exploratory model, female COMT -/- show increased anxiety. No differences in this behavior were reported for males. However, there were differences in aggression in males with heterozygous COMT-deficient male mice showing increased aggressive behavior.

This present experiment was designed to determine the effect of deletion of the COMT gene on cognitive behavior and the stress response in females. To further understand the interactions between genetic alterations in COMT and environmental factors, vulnerability to stress was investigated in female catechol-

o-methyltransferase null mutants (COMT -/-). It was hypothesized that null mutation of COMT, since it primarily affects catecholamines in the prefrontal cortex, will impair learning and memory as assessed by spatial and non-spatial tasks since prefrontal dopamine levels are important in cognition (Goldman-Rakic, 1992). In addition, it is hypothesized that chronic stress will be more detrimental to COMT -/- mice than normal mice because of an inability to return the system to pre-stress homeostasis.

EXPERIMENTAL PROCEDURES

Experiment 3A

General

Catechol-*o*-methyltransferase null mutants (COMT -/-) were generated by a targeting approach described elsewhere (Gogos et al., 1998). Briefly, a human *comt* cDNA probe was used to screen a mouse brain c DNA library. A full-length cDNA clone was isolated and part of it was used to screeen a mouse 129/Sv genomic library. A positive phage encompassing the entire set of *comt* coding exons was isolated and used to prepare the targeting construct. Heterozygous mice showed the expected pattern of gene disruption by Southern and PCR analysis (Gogos, Morgan, et al., 1998). They were viable and fertile and were intercrossed to obtain homozygous COMT-null mutant (COMT -/-) mice.

Thirty-two female mice served as subjects. 10 COMT +/+ and 15 COMT -/- mice, in three cohorts were transported from Rockefeller University and acclimated in the animal facility at Hunter College for one week before testing. All cohorts received the same conditions, and statistical analysis confirmed no

differences between cohorts on any measure. Five subjects were taken out of the analysis because they were male. One subjects was lost to illness and an additional subject had no eyes and was eliminated from testing. In all three cohorts, half the mice from each genotype (total of 8 COMT -/- and 5 COMT +/+ subjects) were restrained in Broome rodent restrainers (diameter 25.4 mm) for 4 hours daily for 14 days (as previously described (Zerbib et al., 1990), Kneavel et al., submitted manuscript). For the first 8 days of the chronic stress, subjects were restrained for 4 hours continuously. After this period, the mice were exhibiting signs of extreme distress and the 4-hour stress period was separated into 2 2-hour periods for the remaining 6 stress days. Subjects in the chronic stress group were restrained in a separate room in sound attenuated box during the stress period to diminish effects of any vocalizations or pheromones on non-stressed controls. Non-stressed controls remained in their home cages and were handled and weighed daily. Weights were analyzed across the stress period with a mixed design, repeated measures ANOVA to assess genotype (COMT +/+ vs. COMT -/-) and stress (control vs. stress) effects across the stress day (0 – 14).

Subjects were maintained on a 12:12 reverse light/dark cycle (lights on 9 AM) and tested during the subjective active period starting at 10 AM and 2 PM. Subjects were double housed during acclimation, stress, open field testing, and singly housed during object recognition testing and radial arm maze testing. Subjects were sacrificed during a delay period on the radial arm maze in a separate room through rapid decapitation. For neurochemical analysis, brains were rapidly removed following decapitation, frozen on dry ice, and stored at -

60°C for later neurochemical analyses. For all behavioral and neurochemical analysis, the primary investigator had a master list of genotype and stress conditions. Others who assisted in gathering behavioral and neurochemical data were blind to condition. All subjects were treated under NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

Open Field

Day 1 post-stress: Subjects were placed on a 45 cm square Plexiglas open field with a 4 x 4 grid for 6 minutes as previously described (Kneavel et al., submitted manuscript, 2001). The behaviors recorded were sector visits, rears, grooms, and defecations. A sector visit was defined as a subject bringing at least half of its torso into a sector (no subject can be in 2 sectors at once). Rearing was defined as a subject raising its upper torso, so that its forelimbs were at least at the position of its head during ambulation. Wall climbs involved the subject raising its torso and placing its front forelimbs on the wall. Between subjects 2 x 2 ANOVAS were used to assess differences between genotype (COMT +/+ and COMT -/-), and stress (non-stress vs. stress).

Object Recognition

Days 1-3 post stress: Object recognition testing consisted of two 5-minute sessions (in the 45 cm square field): a sample trial (T1) and a recognition trial (T2), which were separated by an inter-trial interval of 1 minute, 10 minutes, and 1 hour. Following open field testing (in the afternoon of day 1 post-stress),

subjects were exposed to a 1 minute inter-trial object recognition delay. The next day, a 1-hour inter-trial delay and a 10-minute inter-trial delay took place. On the third day following stress, a second 1-hour delay trial was given.

During T1, two identical objects were placed on the field opposite the experimenter. At the beginning of each 5-minute session, the subject was placed on center of the field facing the objects. Exploration of the objects was timed with stopwatches when the subject sniffed, whisked, investigated, or oriented towards the objects from no more than 2 cm away (Beck et al., 1999; Ennaceur et al., 1994; Ennaceur et al., 1997; Ennaceur et al., 1989). For each subject, the stopwatch was started when the subject began exploration and the stopwatch was stopped when the subject ceased exploration of the object. After the inter-trial delay, the recognition trial (T2) began; one of the objects was replaced with a novel object. Exploration of the novel and old objects was timed with stopwatches. The total time spent exploring both the old and new object were recorded and the percentage of time exploring the new object was analyzed (see (Beck et al., 1998; Ennaceur et al., 1994; Ennaceur et al., 1988; Ennaceur et al., 1989; Ennaceur et al., 1992 for further details). Objects used were determined previously to be equally attractive to mice (unpublished data) and included button covers, small paper binders, sake cups (porcelain and plastic), plastic teacups, wooden napkin rings, and plastic PVC pieces. The object that served as the old object, and its position (left or right) was counterbalanced across subjects on each trial and different objects were used for each different delay trial. Chance performance in this task leads to an even split in the time spent exploring the

objects with an average of 50% exploration of the novel object and 50% exploration of the old object.

Two x two between subjects ANOVAs were used to assesss any pre-existing differences in initial exploration time (T1) between the genotypes (COMT +/+ and COMT -/-) and stress conditions (non-stress and stress) at each delay period. For analysis of object recognition, the percent time spent exploring the new object data was transformed by using the arcsine of the square root of the percentage to normalize the distribution (Sokal et al., 1995; Beck et al., 1999; Ennaceur et al., 1988; Ennaceur et al., 1989; Ennaceur et al., 1992; Ennaceur et al., 1994; Ennaceur et al., 1997). This transformed percentage was then tested with between-subjects 2 (genotype: COMT +/+, COMT -/-) x 2 (stress: non-stress and stress conditions) ANOVAs at each delay period (Beck et al., 1998).

Radial Arm Maze (RAM)

Days 6- 22 post-stress: Twenty-four hours prior to testing, subjects were food deprived to 90- 95% baseline weight and maintained at this weight for the duration of testing on the RAM. Subjects were tested twice a day on the radial arm maze. Subjects were checked daily for dehydration or lethargy, and more food was given if large weight loss was detected.

For the first 10 training/ acquisition trials, the subject is placed on the center of the eight arm maze to explore the arms and find the food reward (peanuts) which was placed at the end of the arm and at 11/3 and 2/3 the length of the arm. The subject explored the maze for 10 minutes or until all eight arms had been visited. A visit to the arm was recorded if the subject traveled more than

half-way down the arm. The total errors (visiting an arm already visited), errors in the first eight choices, and at what choice the first error occurred were recorded for the acquisition trials.

After the first 10 acquisition trials, 5 testing trials began: subjects were again placed on the maze until they visit all eight arms during each testing trial. Again, the total errors (visiting an arm already visited), errors in the first eight choices, and at what choice the first error occurred were recorded.

After the testing trials, subjects were tested on delay trials in which the subject is taken off the maze between the 4^{th} and 5^{th} choice for an inter-trial delay of 10 minutes, 1 hour, 2 hours, and 3 hours. The 10-minute, 1-hour, and 2-hour delay trials were administered twice on the same day. After the delay, the subject was placed back on the maze until all the previously unvisited arms were visited.

Mixed-design repeated measures ANOVAs were performed on all RAM data and Fisher LSD post-hoc analysis was used where appropriate.

Neurochemical analysis

Following completion of all behavioral testing, subjects were placed on the radial arm maze for four arm choices. Immediately following the four arm choices, each subject was taken into a separate room and sacrificed by rapid decapitation (without anesthesia). The brains were quickly removed and immediately placed on dry ice as previously described (Kneavel et al., submitted manuscript, (Beck et al., 1998),(Gogos et al., 1998). Brains were sectioned at 250-300μm at –6to -8 C in a microtome cryostat. Hippocampal, amygdaloid, and frontal cortical brain areas were micropunched from sections according to the

atlas of Franklin and Paxinos with a 500μm diameter cannula while the slide rested on a microscope stage maintained at –11.5 C as previously described by Gogos et al. (1998). Punched samples were placed in 1 ml Eppendorf tubes with 60μl of sodium acetate buffer containing α-methyl-dopamine (as internal standard). After freeze-thawing and centrifugation, the supernatant was removed and 2μl of 1 mg/ml ascorbate oxidase solution (Sigma) was added to each sample to minimize the HPLC front. Forty microliters was injected in a Waters Associates chromatographic system consisting of a 717 Plus autosampler, 590 pump, and C-18 reverse-phase 3-μm Velosep column (Rainin Instruments). An ESA 5011 Coulocomb 3100A electrochemical detector with the screening electrode set at +0.05 V and the detecting electrode at +0.35 V was used. Concentrations of neurotransmitters and metabolites were calculated by reference to standards using peak integration with a computer assisted Waters Millenium system. Sample runs averaged 30 to 40 minutes. The pellet was then dissolved in 100μl of 0.2 N NaOH for protein determination by the Bradford method (Bradford, 1976). Concentrations are expressed as pg/μg protein. Neurochemical data was analyzed using a between subjects 2 x 2 ANOVA to assess differences and interactions between genotype (COMT +/+ and COMT -/-) and stress (non-stress and stress).

Statistical Analysis

All data was analyzed using NCSS (Hintze, 1999). Data were checked to ensure normal distribution using the 3 D'Agostino normality tests for skewness, kurtosis, and omnibus ($p<0.05$) and screened for outliers using a T^2 test based on

Mahalanobis distance of each point from the variable means. Fisher's LSD was used for all post-hoc analyses. Probability for rejecting the null hypothesis was set at $p<0.05$. See individual methods sections for specific analyses.

RESULTS EXPERIMENT 3A

Weight

Subject's weights were monitored throughout stress and behavioral testing. No genotype differences were observed ($F_{(1,22)}=1.85$, $p>0.05$, Fig. 18). Stress, however, did significantly affect weights of both COMT +/+ and COMT -/- subjects across the fourteen day stress periods, $F_{(1,22)}=10.99$, $p<0.005$ (Figure 18). Post-hoc analysis with Fisher's LSD revealed that stressed animals weighed less than non-stressed controls as soon as day 2 of stress and then throughout the stress period.

Open Field

Stress affected COMT +/+ and COMT -/- subjects differently on measures of activity on the open field. A significant interaction between genotype and stress was found in total sector crossings on the open field, $F_{(1,21)}=4.41$, $p<0.05$ (Table 6). Post-hoc analysis of this effect with Fishers LSD ($p<0.05$) revealed that non-stressed COMT -/- subjects made significantly fewer crossings than non-stressed COMT +/+ controls or stressed COMT -/- subjects. A similar interaction was observed in perimeter sector crossings, $F_{(1,21)}=5.90$, $p<0.05$ with the non-stressed COMT -/- subjects making fewer inside sector crossings than other groups (Table 6). These results indicate that COMT -/- subjects were less active but stress increased activity.

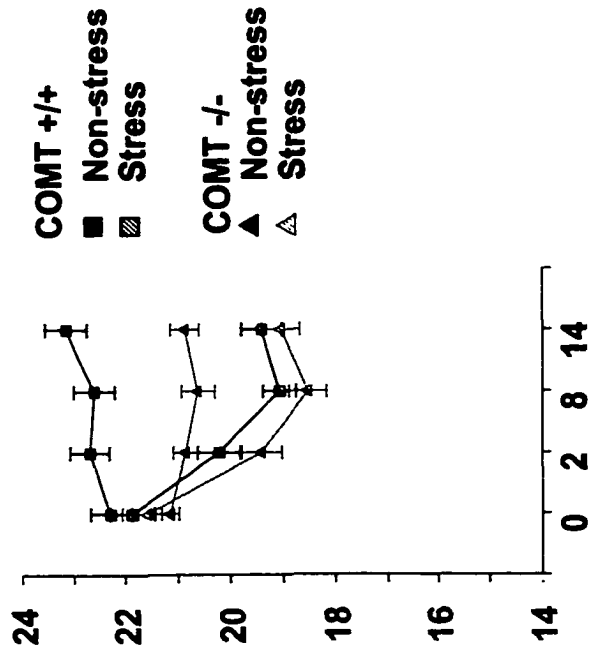


Figure 18

Table 6: Open Field COMT Exp 3A

	COMT +/+		COMT -/-	
	Non-stress (n=5)	**Stress (n=5)**	**Non-stress (n=7)**	**Stress (n=8)**
Total Sector Crossings	218.2 ± 9.8	206.2 ± 7.0	181.7 ± 10.3 [a]	219.4 ± 13.5
Inside Sector Crossings	31.0 ± 8.3	38.4 ± 3.7	34.6 ± 5.3 [a]	42.8 ± 6.6
Perimeter Sector Crossings	187.2 ± 7.2	167.8 ± 7.9	147.1 ± 9.6	176.6 ± 10.8
Ratio Inside/Perimeter	0.17 ± 0.04	0.23 ± 0.02	0.24 ± 0.04	0.25 ± 0.04
Rears	11.2 ± 2.7	15.8 ± 4.9	15.3 ± 3.5	12.5 ± 1.5
Grooms	2.0 ± 1.0	2.5 ± 0.5	1.0 ± 0	2.7 ± 1.2
Wall Climbs	21.0 ± 4.9	25.8 ± 2.9*	14.1 ± 2.6	27.9 ± 1.0*

Activity on the open field in male and female COMT +/+ and COMT -/- non-stressed and stressed subjects. Asterisk (*) indicates a significant stress effect in which stressed subjects of both genotypes had more wall climbs than non-stressed subjects (*$p<0.01$). Symbol ([a]) indicates a signficant genotype by stress interaction ($p<0.05$). COMT -/- non-stressed subjects had the fewest total sector crossings and perimeter sector crossings ([a] $p<0.05$).

Wall climbs, which are generally thought of as escape behaviors, and therefore expected to increase with stress exposure, were significantly higher in stressed subjects of both genotypes. Stressed subjects had significantly more wall climbs (27.1 ± 1.9) while on the open field than non-stressed subjects (17.0 ± 2.0), $F_{(1,21)}$=11.04, p<0.005, Table 6.

Object Recognition

Analysis of the time spent exploring the 'old' objects in T1, a measure of potential attention or locomotor differences, revealed no significant genotype, stress or interactions at any delay period, Figure 19. Specifically, with an inter-trial delay of 1 minute, there was no difference in exploration time at T1 between genotypes ($F_{(1,25)}$=0.32, p>0.05) or stress ($F_{(1,25)}$=4.20, p>0.05) or any interactions ($F_{(1,25)}$=0.07, p>0.05), Figure 19. With a 10-minute inter-trial delay, there were no differences in exploration time at T1 between genotypes ($F_{(1,25)}$=0.01, p>0.05) or stress ($F_{(1,25)}$=3.46, p>0.05) or any interactions ($F_{(1,25)}$=0.38, p>0.05), Figure 19. With a 1-hour inter-trial delay, there were no differences in exploration time at T1 between genotypes ($F_{(1,24)}$=0.98, p>0.05) or stress ($F_{(1,24)}$=1.50, p>0.05) or any interactions ($F_{(1,24)}$=0.05, p>0.05), Figure 19.

Many of the subjects did not extensively explore the objects during T2. Previous studies utilized only subjects which spent more than 5 seconds exploring both objects. Because of this requirement, very few subjects were included in the analysis, and accurate assessment of object recognition was impractical. For the 1 minute delay, 2 non-stressed COMT +/+, 1 stressed COMT +/+, and 1 non-stressed COMT -/- were eliminated from analysis because they spent less than 5

Figure 19

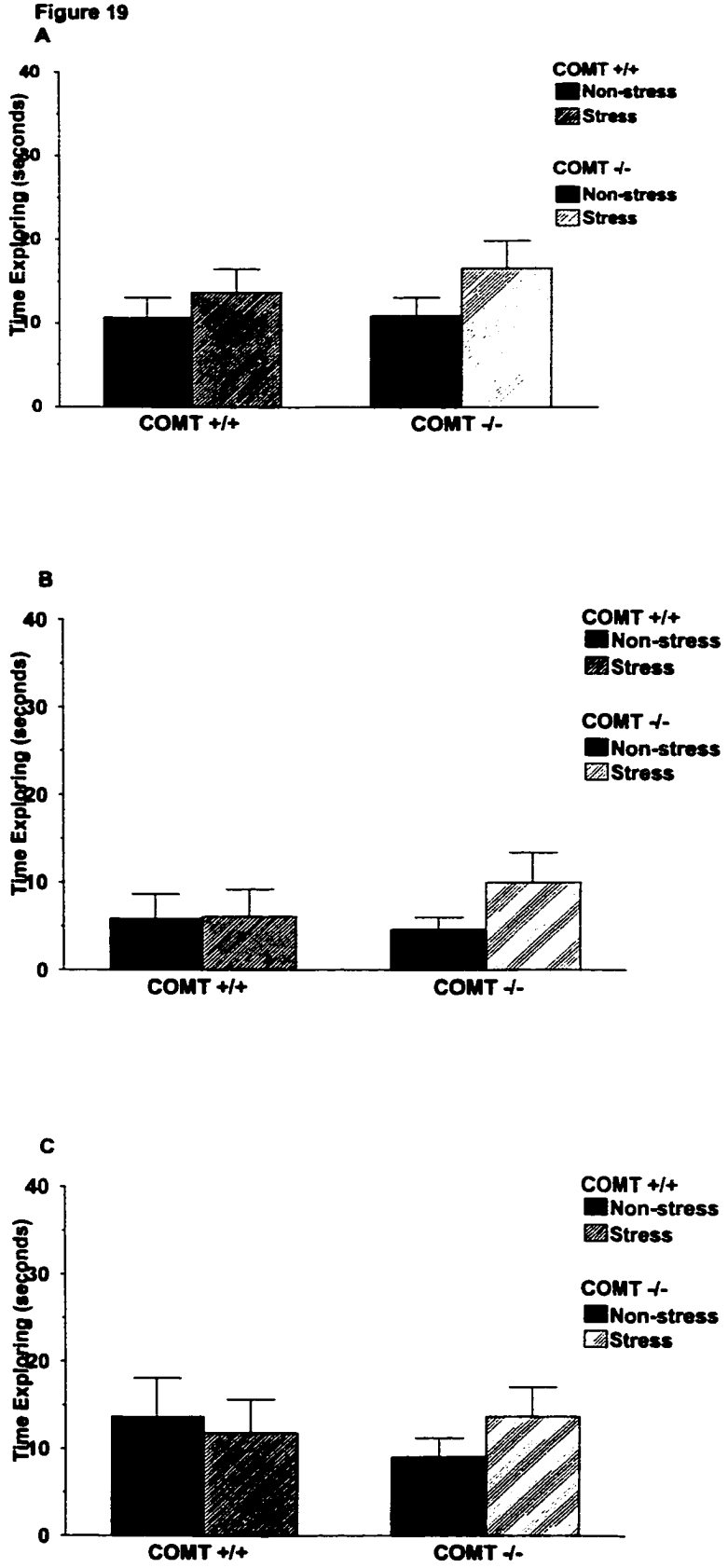
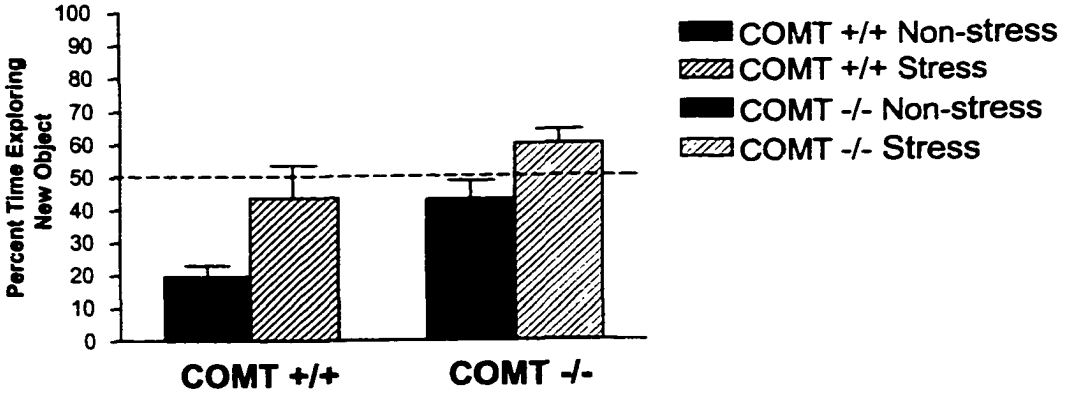


Figure 20

A



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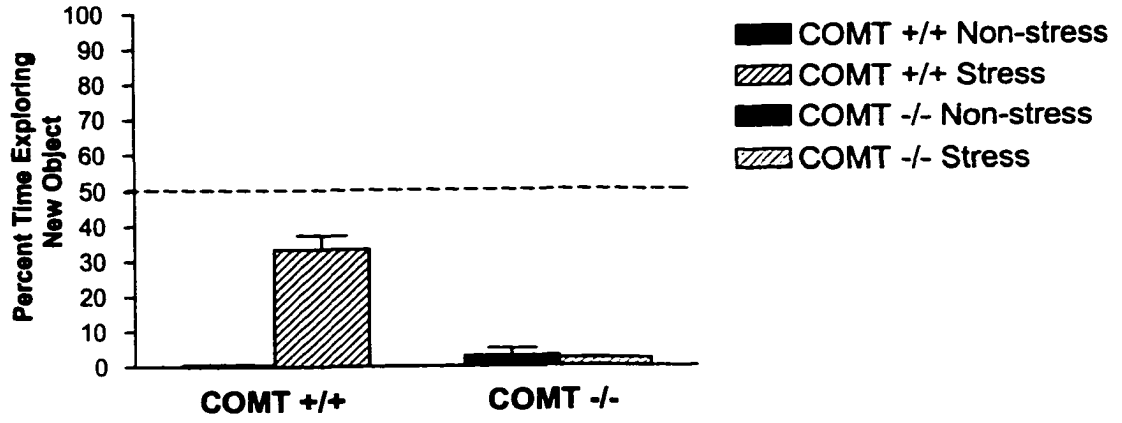

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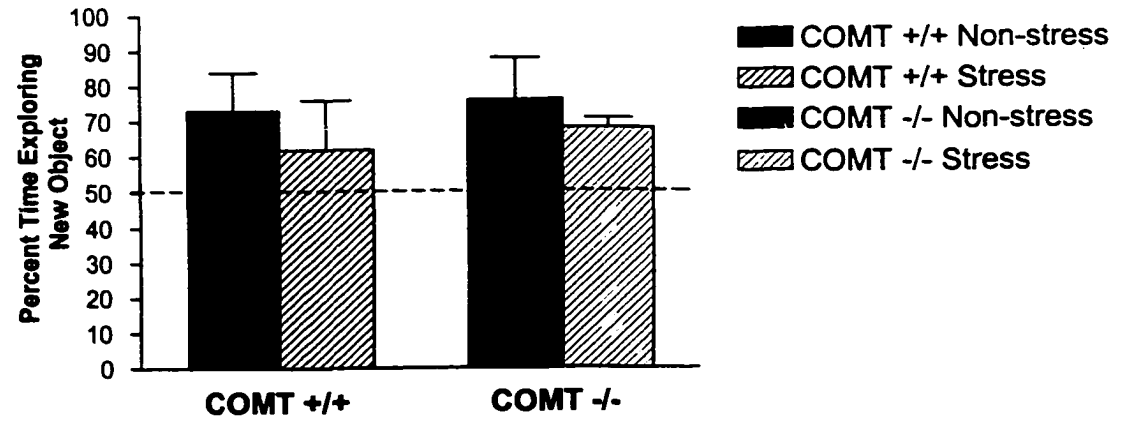


seconds exploring the objects. For the 10 minute delay, 2 non-stressed COMT +/+, 3 stressed COMT +/+, 4 non-stressed COMT -/-, and 3 stressed COMT -/- subjects did not explore the objects for more than 5 seconds. At the 1 hour delay, 3 non-stressed COMT +/+, 1 stressed COMT +/+, 3 non-stressed COMT -/-, and 1 stressed COMT -/- subjects were eliminated from analyses because they explored the objects for less than 5 seconds. With these limits, no significant genotype, stress, or interaction effects were observed at any of the delays, Figure 20.

Radial Arm Maze (RAM)

Testing Trials (RAM)

There were no significant differences in the amount of time to complete the radial arm maze between COMT +/+ and COMT -/- ($F_{(1,25)}$ =0.02, p>0.05) or stress and control subjects ($F_{(1,25)}$=0.27, p>0.05), Figure 21. There were no significant main effects for genotype, however there were significant effects of stress across testing trials. Specifically, there was a significant stress by trial effect on the first error choice, $F_{(4,79)}$= 2.83, p<0.05 in which post-hoc analysis revealed that all stressed subjects, COMT +/+ and COMt -/-, are making the first mistake later than non-stressed subjects as the trials increase from 1 to 5 (data not shown). In addition, there were no signficant genotype ($F_{(1,21)}$ = 0.35, p>0.05) or stress ($F_{(1,21)}$=0.14, p>0.05) effects in total errors, Figure 22. However, a significant trial effect on the number of correct choices in the first eight arm visits confirmed that all subjects improved as trials increased, $F_{(4,44)}$=3.43, p<0.05, Figure 23. Post-hoc

Figure 21

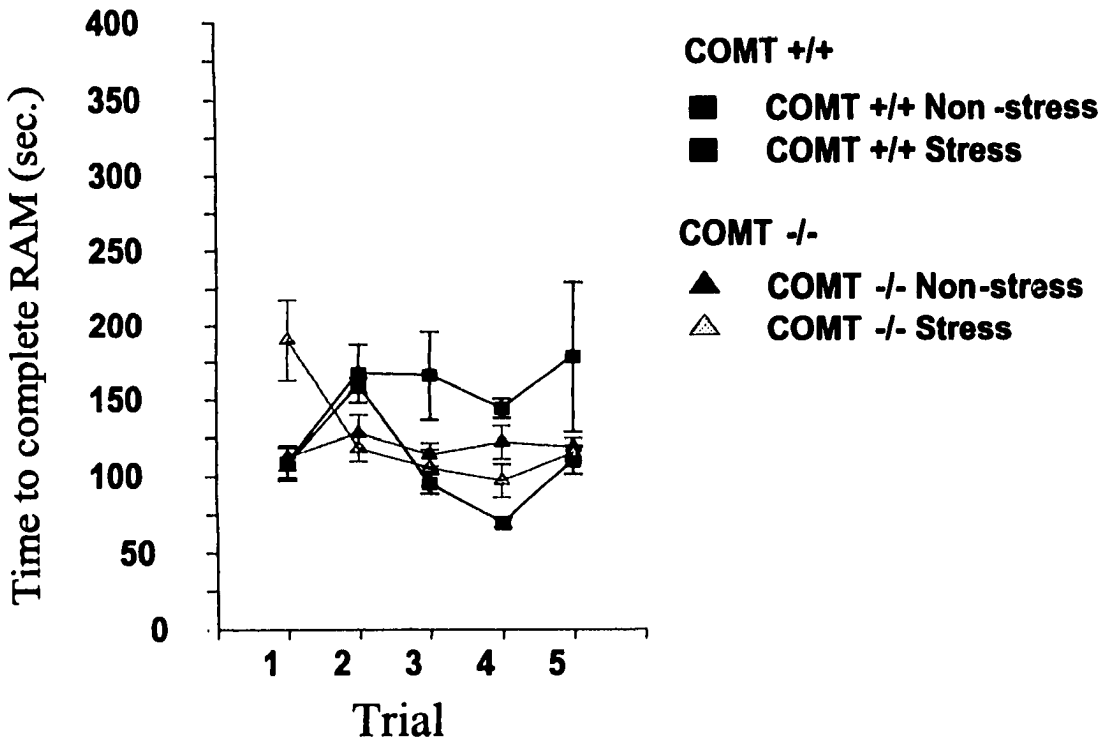


Figure 22

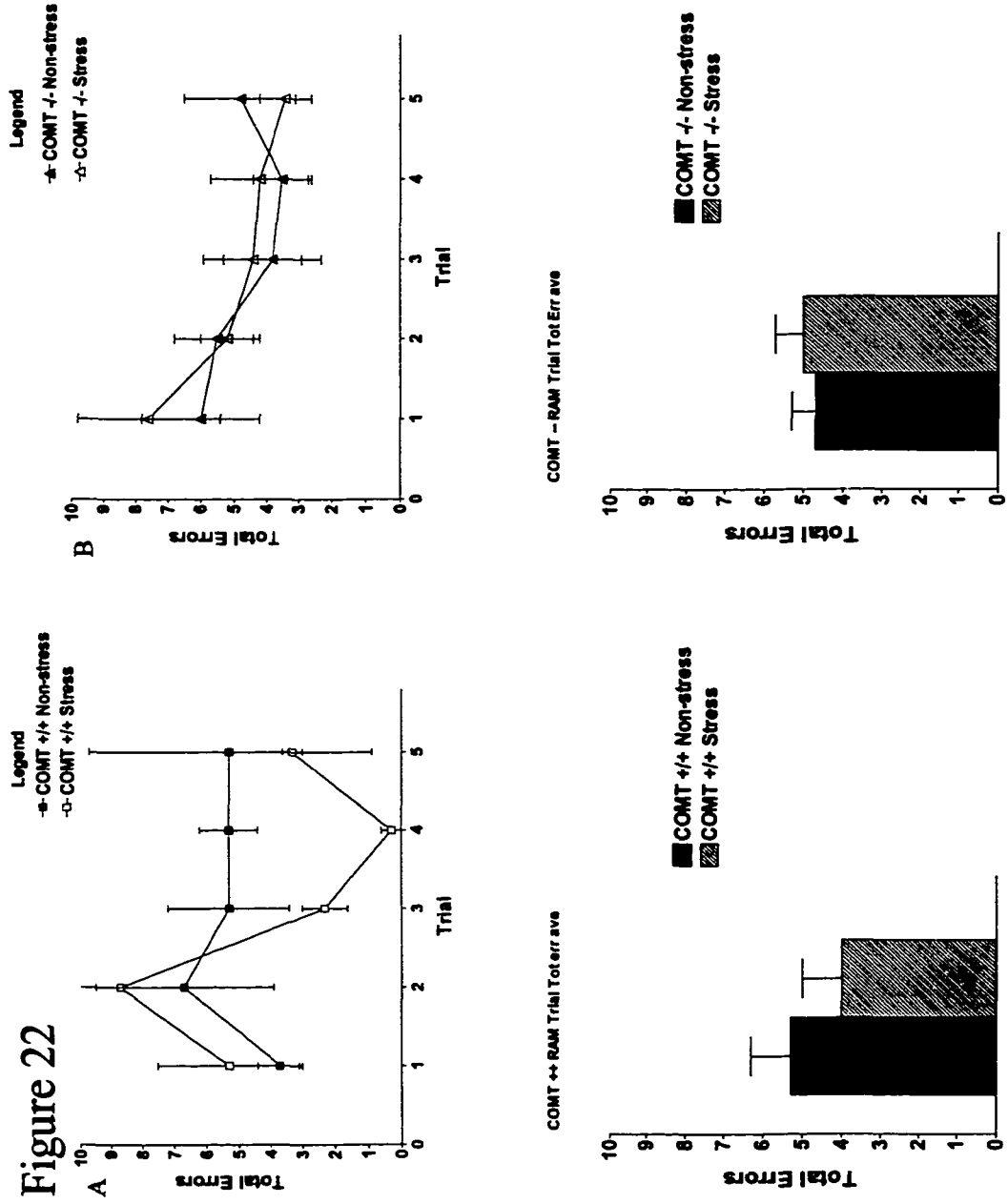


Figure 22

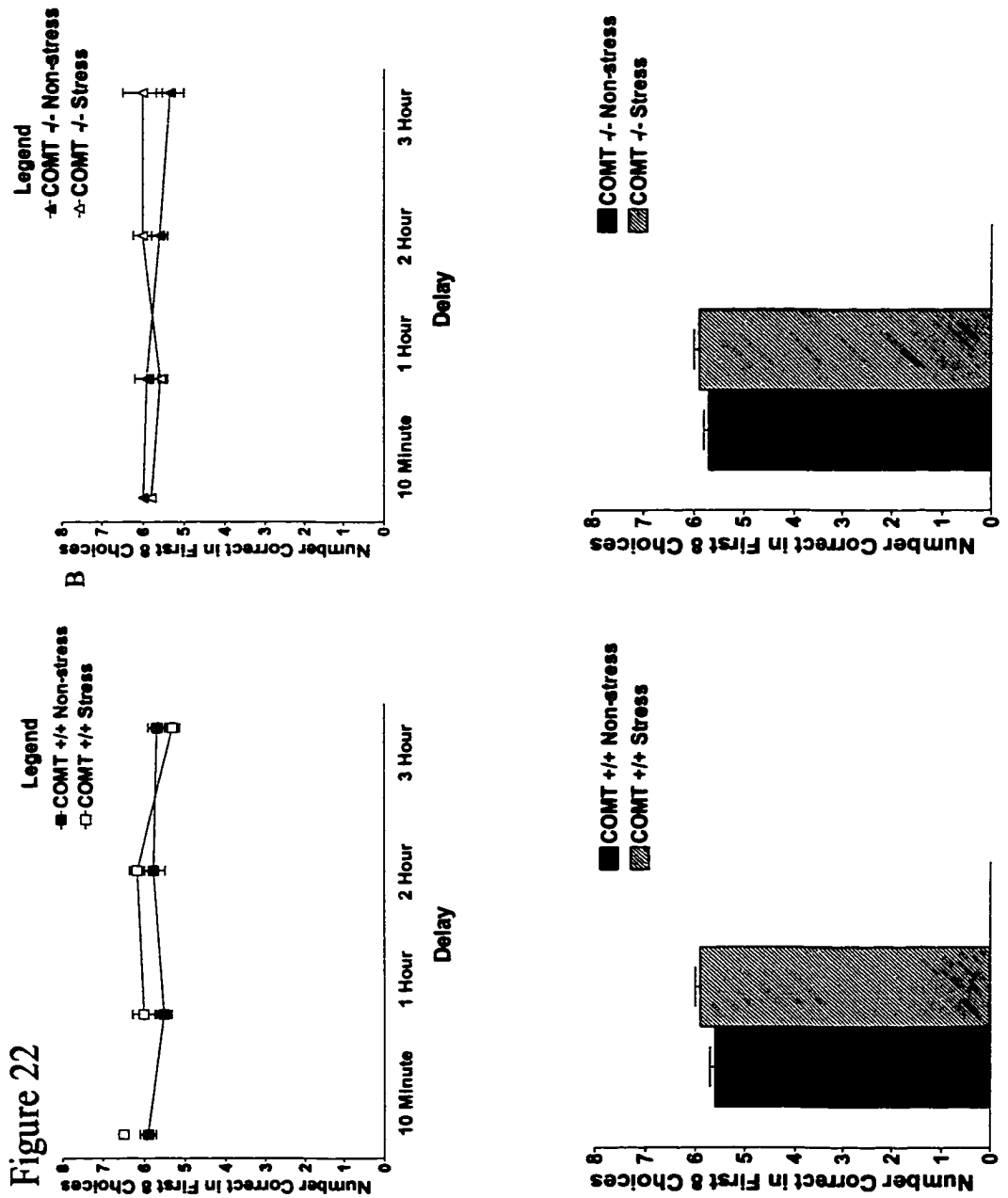
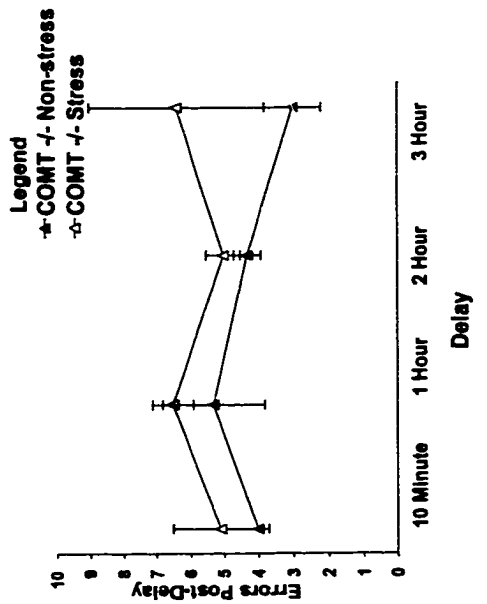
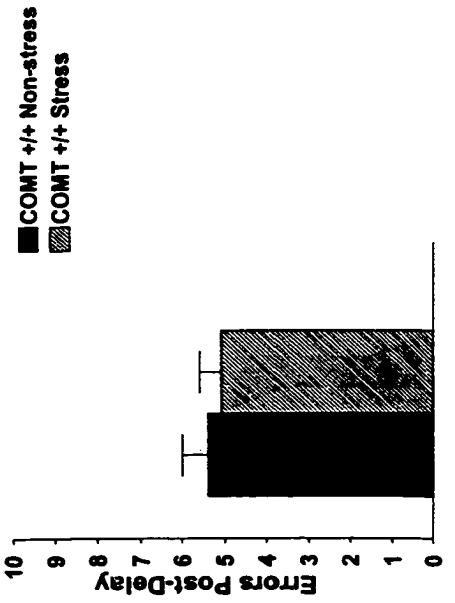
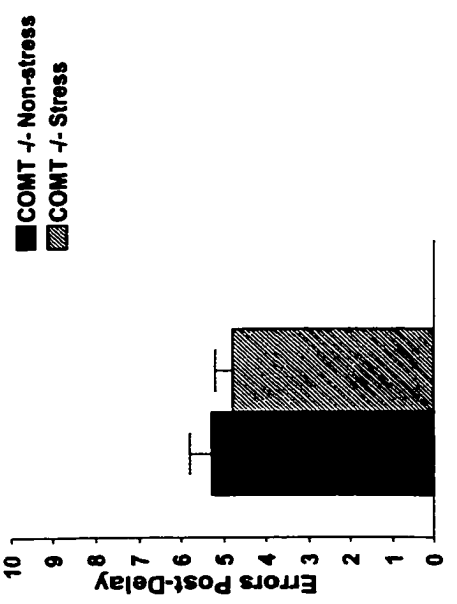


Figure 24







analysis of this measure with Fisher's LSD test ($p<0.05$) indicates that trials 3, 4, and 5 are significantly different from the early trials 1 and 2, Figure 23.

Delay Trials (RAM)

As more cognitive demand was introduced into the radial arm maze task by placing delays between the fourth and fifth arm choice, stressed subjects continued to perform differently than non-stressed subjects across the delays. A marginally significant stress by delay period effect on total errors, $F_{(3,33)}=2.83$, $p=0.053$, Figure 24 was found and subsequent post-hoc analysis revealed that stressed subjects made fewer total errors at the 3 hour delay than non-stressed subjects.

Neurochemistry

No significant genotype, stress, or interactions were observed in neurochemistry in the frontal cortex, amygdala, or hippocampus.

BRIEF DISCUSSION EXPERIMENT 3A

Data from the testing trials and delay trials point to the conclusion that fourteen-day restraint stress may enhance memory in both COMT +/+ and COMT -/- as assessed on the radial arm maze. Further, COMT deficiency does not severely impair subjects in this spatial memory task and stress was as enhancing for this group as for the wild-type (COMT +/+) subjects. One of the reasons the stress did not significantly enhance performance during the testing trials may be due to a drop-off in performance of all subjects on the last trial. Another explanation may be that the enhancing effects of stress do not become apparent until the task becomes more difficult. It is possible that the regular trials

of the radial arm maze are easy for all subjects and thus no differences were present between groups. These data are similar to findings with male rats who perform better after fourteen days of stress and worse after twenty-one days compared to non-stressed controls (Luine, et al., 1994) and to female rats who show enhanced radial arm maze performance following 21-days of restraint (Bowman and Luine 2001). Unfortunately, no useful results were obtained from the object recognition task since most subjects did not spend sufficient time exploring the objects. Previous reports in rats have found that females spent significantly less time exploring objects compared to males (Beck and Luine, submitted manuscript). As previously reported, there were no significant differences in neurochemistry of these COMT -/- females. Somewhat surprisingly, no significant stress or interaction effects were found. However, the lack of robust stress effects on the radial arm maze predicted a similar lack of change in monoamines since previous results have shown stress dependent changes in monoamines which are related to changes in radial arm maze performance (see Kneavel et al., submitted manuscript; Luine, Villegas, Martinez, & McEwen, 1994b).

Experiment 3B: Effect of COMT deletion in female mice on 129sve background strain.

INTRODUCTION

Overall strain differences in reactivity to different drugs and environmental conditions exist and can have a profound impact on the behaviors

being measured. Further, even within the confines of a particular construct, such as memory, strain differences can vary widely. Results presented previously indicated that there are significant differences in performance between the C57BL/6 and 129Sve strains on behaviors used to assess memory function and overall activity such as open field, object recognition, and radial arm maze (Kneavel et al., submitted manuscript). These strain differences are task dependent. On the open field, for instance, C57BL/6 have been found to be more active (Homanics et al., 1999; Kneavel et al., submitted manuscript). Additionally, differences in baseline performance between the strains on both the radial arm maze and object recognition tasks exist. Previously reported results of radial arm maze performance confirm that C57BL/6 perform better than 129Sve (Nguyen et al., 2000; Kneavel et al, 2001). In contrast to the radial arm maze, 129Sve perform very well on the object recognition task. 129Sve are discriminating between the old and the new objects at delays up to 3 hours. In contrast, C57BL/6 subjects discriminate up to a one-hour delay and are unable to discriminate at the longer 2 and 3-hour delays. These results support the idea that better performance on the radial arm maze in C57BL/6 subjects is not necessarily due to differences in memory but due to differences in requirements for performing the task. Since the object recognition task relies less on ambulation than the radial arm maze and does not require food restriction, the object recognition task may be a better tool for assessing memory in mice.

Genetic differences between parental strains used for the creation of transgenic and COMT -/- mice may also influence the phenotype under

investigation. If genetic differences are linked to the targeted mutation, genetic polymorphisms can cosegregate and influence the phenotype and lead to an incorrect assessment of the influence of the targeted gene mutation alone. Additionally, the background strain on which the genetic mutation is maintained can interact with the targeted gene mutation and influence phenotype. This study investigates the influence of the COMT deletion on a 129Sve background strain by comparing open field, object recognition, and radial arm maze performance in COMT +/+ and COMT -/- mice maintained on a 129Sve background by comparing open field, object recognition, and radial armmaze in COMT -/- and COMT +/+ mice on a 129Sve background.

EXPERIMENTAL PROCEDURES

EXPERIMENT 3B

General

Generation of the COMT null mutants for this experiment was similar to that described above except the null mutation was maintained on the 129Sve background. Twenty female mice served as subjects. COMT129 -/- and COMT129 +/+ subjects were generated and tested at Rockefeller University. Subjects were housed on a 12:12 reverse light/dark cycle (lights off 9 AM) and tested during the subjective active period under dim red light conditions starting at 10 AM. Subjects were single housed throughout behavioral testing on the open field, object recognition, and radial arm maze. At the end of behavioral testing, subjects were sacrificed during a delay period on the radial arm maze in a separate room through rapid decapitation.

Open Field

As previously described, subjects were placed on a 45 cm square Plexiglas open field with a 4x 4 grid for 6 minutes. The behaviors recorded were sector visits, rears, grooms, and defecations. A sector visit was defined as a subject bringing at least half of its torso into a sector (no subject can be in 2 sectors at once). Rearing was defined as a subject raising its upper torso, so that its forelimbs are at least at the position of its head during ambulation. Wall climbs involved the subject raising its torso and placing its front forelimbs on the wall. Between subject ANOVAS were used to assess differences between genotypes (COMT 129 +/+ vs. COMT129 -/-).

Object Recognition

Object recognition testing consisted of two 5-minute sessions (in the 45 cm square field): a sample trial (T1) and a recognition trial (T2), which were separated by an inter-trial interval of 10 minutes, 1 hour, and 2 hours. During T1, two identical objects were placed on the field opposite the experimenter. At the beginning of each 5-minute session, the subject was placed on center of the field facing the objects. Exploration of the objects was timed with stopwatches when the subject sniffed, whisked, investigated, or oriented towards the objects from no more than 2 cm away (Beck et al., 1999; Ennaceur et al., 1994; Ennaceur et al., 1997; Ennaceur et al., 1989). For each subject, the stopwatch was started when the subject began exploration and the stopwatch was stopped when the subject ceased exploration of the object. After the inter-trial delay, the recognition trial (T2) began; one of the objects was replaced with a novel object. Exploration of

the novel and old objects was timed with stopwatches. The total time spent exploring both the old and new object were recorded and the percentage of time exploring the new object was analyzed (see (Beck et al., 1998) (Ennaceur et al., 1994; Ennaceur et al., 1988; Ennaceur et al., 1989; Ennaceur et al., 1992) for further details). Objects used were determined previously to be equally attractive to mice (unpublished data) and included button covers, small paper binders, sake cups (porcelain and plastic), plastic teacups, wooden napkin rings, and plastic PVC pieces. The object that served as the old object, and its position (left or right) were counterbalanced across subjects on each trial and different objects were used for each different delay trial. Chance performance in this task leads to an even split in the time spent exploring the objects with an average of 50% exploration of the novel object and 50% exploration of the old object.

Radial Arm Maze (RAM)

Twenty-four hours prior to testing, subjects were food deprived to 90-95% baseline weight and maintained at this weight for the duration of testing on the RAM. Subjects were tested twice a day on the radial arm maze. Subjects were checked daily for dehydration or lethargy, and more food was given if large weight loss was detected. During training, the subject was placed on the center of the eight arm maze to explore the arms and find the food reward (peanuts) which was placed at the end of the arm and at 1 1/3 and 2/3 the length of the arm. The subject explored the maze for 20 minutes or until all eight arms had been visited. A visit to the arm was recorded if the subject traveled more than half-way down the arm. The total errors (visiting an arm already visited), errors in the first eight

choices, and at what choice the first error occurred were recorded for the acquisition trials.

Several subjects failed to visit all eight arms within the twenty-minute time limit on repeated trials and therefore, analysis of total error to complete the task was unattainable. Additionally, delay trials of the radial arm maze were not possible with this group of subjects since several of the subjects never visited all eight arms during a testing trial. Repeated measures ANOVAs were performed on all the choice of the first error and the number of errors in the first eight choices data and Fisher LSD post-hoc analysis was used where appropriate.

RESULTS EXPERIMENT 3B

Open Field

No significant genotype differences were observed on open field activity. Inside visits: $F_{1,18}$=1.48, p>0.05; perimeter crossings: $F_{1,18}$=2.95, p>0.05; rears: $F_{1,18}$=1.41, p>0.05; wall climbs: $F_{1,18}$=1.90p>0.05; grooms: $F_{1,18}$=0.29, p>0.05; or fecal boli: $F_{1,18} = 1.46$, p>0.05, Table 7.

Object Recognition

No significant differences were found in initial exploration of the objects during T1 at any of the delays, Figure 25.

Analysis of the time spent exploring the novel object revealed no significant differences between COMT129 +/+ and COMT129 -/- subjects at the ten minute ($F_{(1, 18)}$= 1.04, p>0.05) or 1 hour delay ($F_{1, 18} = 0.00$, p>0.05). However, at a delay period of 2 hours, COMT129 +/+ subjects spent significantly more time exploring the novel object than COMT129 -/- subjects ($F_{(1, 15)} = 5.43$,

$p<0.05$). At the two hour delay period, COMT129 +/+ subjects are spending 75.7% of the time exploring thenovel objects, whereas, COMT129 -/- subjects are spending 48.4% (chance level), Figure 26.

Radial arm maze

On obtainable measures, a significant trial effect emerged. There was a significant trial effect in the number correct in the first eight choices, $F_{(6,83)} = 2.96$, $p<0.05$, Figure 27. Post-hoc analysis with Fisher's LSD ($p<0.05$) revealed that trials 1, 2, 3, 4, and 6 were different from 7. For the choice of the first error, a marginally significant trial effect indicated a difference between initial trials and later trials ($F_{(6,101)}=2.15$, $p<0.06$). Post-hoc analysis ($p<0.05$) revealed that performance at trials 1, 4, and 5 was significantly different from performance at trial 7, data not shown.

The only genotype difference that emerged was a significant genotype by trial effect ($F_{(6, 83)} = 3.36$, $p<.01$) on the number correct in the first eight choices during testing trials of the radial arm maze, Figure 27. Post-hoc analysis with Fisher's LSD ($p<0.05$) indicates a difference between the genotypes at the first testing trial and none thereafter.

Additional analysis was not possible since several of the subjects did not visit all eight arms within twenty minutes. Specifically, out of the 77 trials that COMT129 -/- subjects were tested on, on only 26 trials did subjects visit all eight arms in 20 minutes and out of 63 trials that COMT 129 +/+ subjects were tested on, on only 28 trials did subjects visit all eight arms and finish the task within the

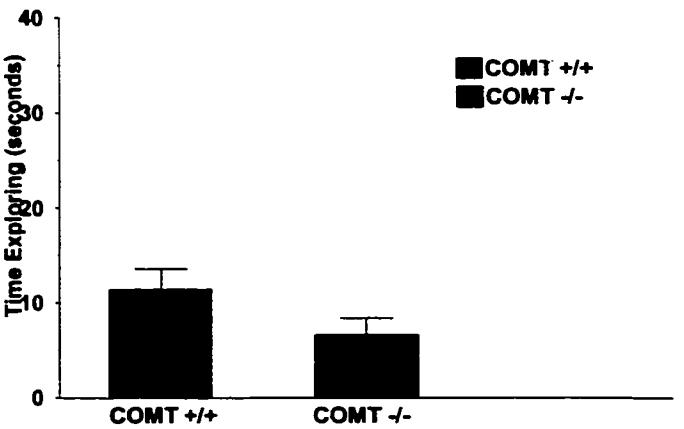
Table 7 : COMT129 Open Field

	COMT +/+	COMT -/-
	Non-stress (n=11)	**Non-stress (n=8)**
Total Sector Crossings	41.7 ± 11.4	78.0 ± 19.5
Inside Sector Crossings	2.6 ± 0.9	5.1 ± 2.1
Perimeter Sector Crossings	39.1 ± 10.7	72.9 ± 17.8
Rears	0	0.1 ± 0.1
Grooms	0.7 ± 0.4	1.0 ± 0.3
Wall Climbs	0.4 ± 0.2	2.8 ± 2.0

No significant differences were found between COMT +/+ and COMT -/- subjects maintained on the 129Sve background on any measures of the open field.

Figure 25

A



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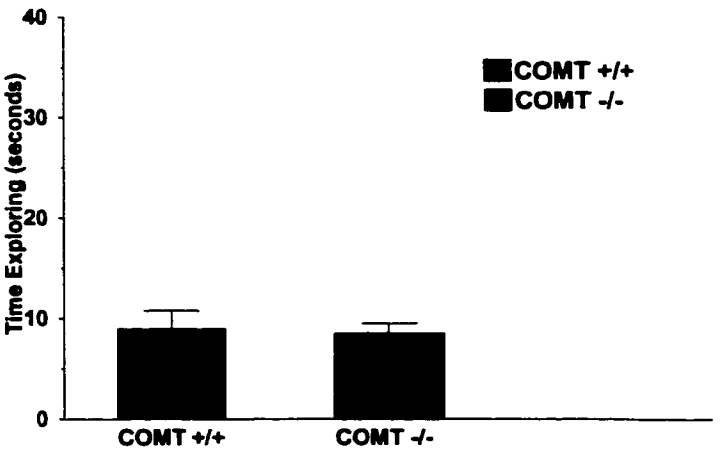

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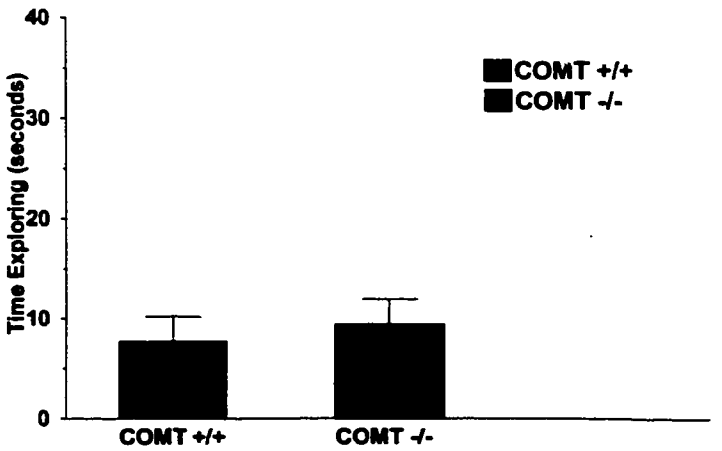
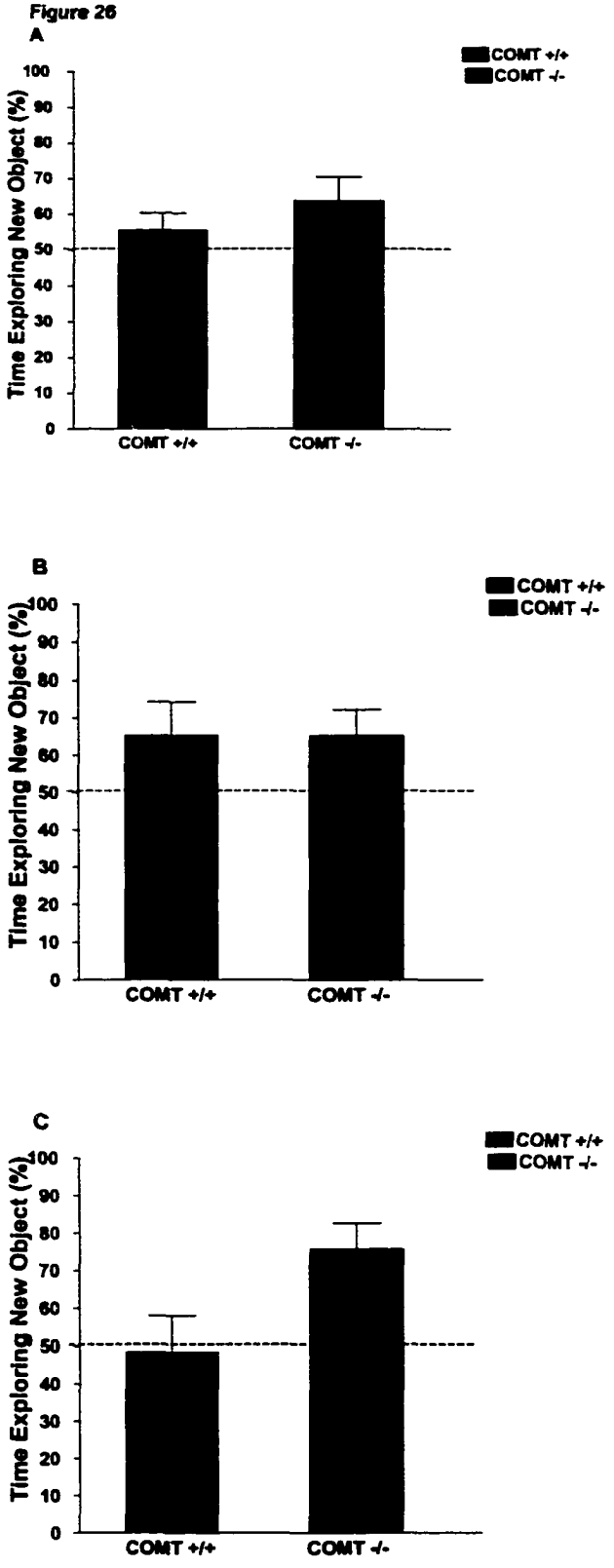


Figure 26



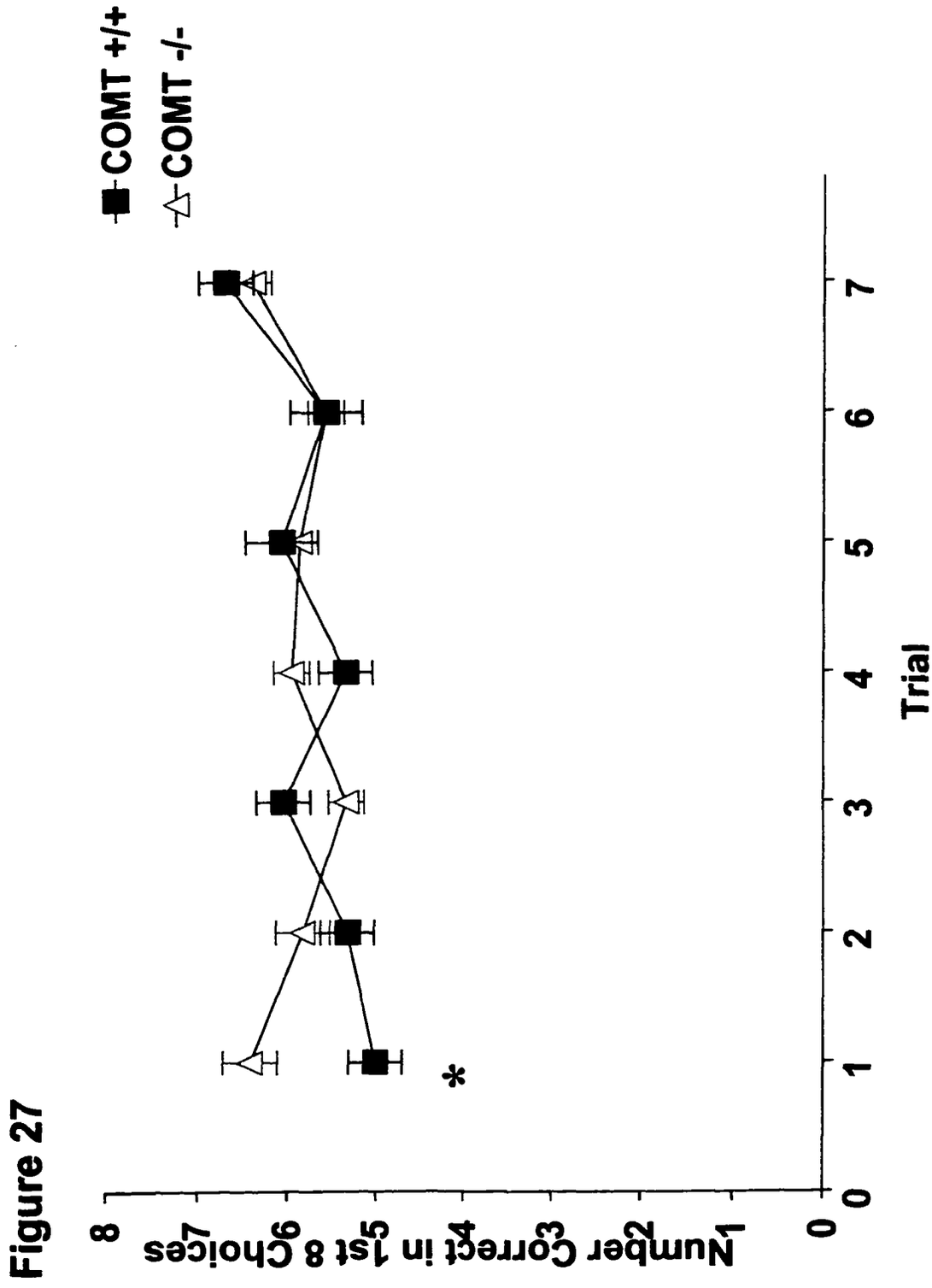


Figure 27

twenty minutes. Analysis of the pattern of missing values or incomplete testing trials using χ^2 test for independence revealed that completion of the task was independent of genotype for the choice of the first error χ^2 (1, n=140) = 0.43, p>0.05, the number correct in the first eight choices χ^2 (1, n=140) = 2.08, p>0.05, and total errors χ^2 (1, n=140) = 1.66, p>0.05. Thus, there was no genotype effect for non-completion trials

BRIEF DISCUSSION EXPERIMENT 3B

Contrary to findings in experiment 3A, some genotype differencess were observed between COMT null mutants and wild-type controls when the mutation was maintained on the 129Sve background. COMT129 subjects in experiment 3B did explore the objects in the object recognition task and it was found that COMT129 +/+ spent more time exploring the novel object at the longest delay interval of 2 hours. While analysis of the radial arm maze data was very difficult and confounded because a large number of both COMT129 +/+ and COMT129 -/- subjects could not complete the task, a small difference in the number correct in the first eight choices was found between the genotypes indicating that on the first testing trial, COMT 129 +/+ had more correct choices.

DISCUSSION

These studies have shown that the genetic background on which a null mutation is maintained can have a profound impact on the expression of the genotype. Not only are the general characteristics of the parent strain evident, but also an interaction between the null mutation and the genome of the parent strain can affect phenotype. These experiments illustrate the importance of having

familiarity with the phenotype of the background strains and the importance of obtaining full understanding of the interaction of the null mutation with a variety of genomic backgrounds. It is necessary to separate the specific effects of the null mutation from the expression of the chimeric backgrounds. In addition, the phenotypic expression of a null mutation may be very different depending on the set of genomic conditions on which it is incorporated.

In these experiments, very different findings were obtained in null mutants maintained on the C57BL background compared to the 129Sve background. Most notably, each of these null mutants could not perform one of the memory tasks. It was not possible to adequately collect object recognition data in the COMT subjects maintained on the C57BL background. On the other hand, while COMT129 subjects, maintained on the 129Sve background, performed very well on the object recognition task, data was unobtainable for the radial arm maze, particularly delay trials. In addition to differences in ability to perform the memory tasks, wild-type controls from each study were also very different in baseline performance on the open field. COMT +/+ on the C57BL background were almost three times more active than COMT129 +/+. This finding is consistent with previous reports of a 2.5 to 3 fold difference in activity between C57BL/6J strains and 129SvEvTac strains on the open field (Kneavel et al., 2001; Homanics et al., 1999; Paulus et al., 1999).

Each background strain provides important information about the role of COMT. The first study provides information about this protein in the stress response. COMT -/- on the C57 background were less active on the open field,

whereas stress returns these COMT -/-s to control levels of ambulation. There were no significant differences between groups in acquisition of the spatial radial arm maze task, however, as the task became more cognitively demanding during delay trials and specifically at the three hour delay between the fourth and fifth arm choice, the stressed subjects, both COMT +/+ and COMT -/- made significantly fewer errors than other groups. Contrary to predictions, the data indicates that COMT deficiency does not severely impair subjects in spatial memory and stress does not further impair performance. In fact, stress enhanced performance of COMT -/- similarly to the enhancement of stress in the COMT +/+. This stress enhancement in COMT +/+ controls corroborates previous findings that fourteen-day restraint stress enhances memory performance in rodents (Kneavel et al., 2001; Luine et al., 1994a). Further testing at longer delays may clarify the role of this enzyme in the stress response cascade since longer delays may push the limits of the memory system in all subjects.

Contrary to predictions, there were no differences in monoamine or metabolite levels in the frontal cortex, hippocampus, or amygdala. However, the lack of large differences in the behavioral data indicated that there would be few if any changes in monoamine systems. These results indicate that in addition to the null mutation not affecting monoamine levels in female COMT -/- (Gogos et al., 1998), the extra insult of environmental stress does not alter monoamines either. Thus, using available methodology, COMT -/- females maintain homeostasis in response to the null mutation and stress and fail to show changes in catecholamine levels or turnover. This is in contrast to male COMT -/- subjects

which do exhibit significant increases in dopamine levels in the frontal cortex and changes in HVA/DOPAC ratios (an indication of decreased turnover and catabolism by COMT) reported earlier (Gogos et al., 1998). Thus, the behavioral enhancement of stress in these females and lack of changes in catacholamines may be due to compensation or protection that is either interacting with or a direct result of female hormones. Further research with ovariectomized (OVX) COMT -/- females and OVX COMT -/- females with hormone replacement may elucidate the role of the female hormones in the protection against the null mutation as well as stress.

Previously reported (Gogos et al., 1998), sexually dimorphisms in COMT -/- established that males and females have different reactions to the deletion. This study provides further evidence that female hormones may protect COMT -/- females not only from deletion of COMT but also environmental stress. In our study, female COMT -/- subjects were not significantly impaired on the spatial radial arm maze and stress did not further impair memory performance, in fact, stress had a slight enhancement effect on performance. However, a significant interaction between stress and genotype on the open field confirms that stress does have a somewhat different effect in female COMT -/- subjects compared to female COMT +/+ subjects. This study found that stress increased ambulation in COMT -/- subjects to the level of COMT +/+ subjects. These results relate to prior reports of anxiety like behavior in female COMT -/- subjects. Similar to the female COMT -/- subjects in this study, it has been previously found that COMT -/- females had decreased ambulation in the light portion of the light/dark shuttle

box, a finding which may reflect alterations in anxiety as well as differences in ambulation (Gogos et al., 1998). To diminish anxiety in our experiment, all subjects were handled daily before behavioral testing began and testing was done under dim red light conditions. Even with these controls, however, results from the open field, mimic results found previously in anxiety tests that decreased activity in female COMT -/- subjects may be related to increased anxiety. Interestingly, stress attenuated this anxiety-like decrease in ambulation in the COMT -/- subjects, which may reflect the common reaction to stress of general increases in activity level (Beck and Luine, 1997). Further, multiple exposure to the radial arm maze during training before testing began may have diminished and possibly eliminated any anxiety or motor related differences not detected on the open field which would have affected performance. This finding is similar to reports that repeated testing could attenuate or eliminate strain differences in motor coordination (Homanics et al., 1999). Thus, it is not surprising that performance was not different in COMT -/- subjects from COMT +/+ subjects on the radial arm maze.

Recent studies using pharmacological inhibition of COMT provides some additional insight into the role of this enzyme in memory processes. Specifically, tolcapone, a centrally acting COMT inhibitor, decreased the number of errors on the radial arm maze in adult rats (Liljequist R, Haapalinna A, Ahlander M, Li, & Mannisto, 1997) and has been shown to counteract the damaging effects of scopolamine on passive avoidance memory (Khromova, Voronina, Kraineva, Zolotov, & Mannisto, 1997). In addition, there is some evidence that COMT

inhibition protects memory function in cholinotoxin lesions (Khromova, Rauhala, Zolotov, Mannisto, 1995). While tolcapone does not consistently improve memory or protect against neuronal injury, previous reports indicate that COMT inhibition does seem to have protective and memory enhancing properties, particularly at higher doses and on spatial memory tasks (Liljequist, et al., 1997, Khromova, et al., 1997, Khromova, et al., 1995). These results collectively support our findings that COMT -/- were not impaired on memory tasks and were able endure stress.

Lack of changes in catecholamine or metabolite levels in COMT -/- mice is consistent with findings in studies of similar null mutants. Evidence from monoamine oxidase B knockout mice indicates that while no significant changes in extracellular dopamine levels, synthesis, storage, or release of dopamine exist, significant upregulation of D2-like dopamine receptors and supersensitivity of D1-dopamine receptors did occur (Chen et al., 1999). No information is currently available on changes in dopamine or norepinephrine receptor expression in COMT -/- subjects. Further investigation of expression of both receptors in COMT -/- female and stressed mice is needed to understand the relationship between COMT, stress, and female hormones.

In addition to proposed experiments mentioned above, further understanding of the mechanisms of COMT could be ascertained through a more comprehensive assessment of the influence of the COMT enzyme specifically through inclusion of male subjects. Previous work with males and females has shown significant differences on several behavioral tasks and there may be similar

sex differences in learning and memory and stress reactivity. If females are less effected by the null mutation than males consistent with neurotransmitter differences, this may point to a compensation mechanism in females useful for combating the effects of COMT deficiency. In addition, future studies on the direct effect of stress on neurochemical levels in these COMT -/-s through in vitro dialysis or measurement of levels directly after stress (without intervening behavioral testing) may provide further insight into the mechanisms of COMT, dopamine, norepinephrine, epinephrine, metabolites, and frontal cortex functioning in the stress response.

Additional evidence for the role of COMT and the interaction with genetic background is provided in the second study. Results from both studies indicate that the phenotypic expression of the COMT deletion in females can be strongly influenced by the background strain on which the null mutation is maintained. Specifically, differences between COMT +/+ and COMT -/- subjects maintained on the C57BL/6 background were not necessarily apparent in COMT +/+ and COMT -/- subjects maintained on the 129Sve background. One of the main difficulties in interpreting the results of course is the lack of data from either the object recognition or radial arm maze task in each experiment. However, although difficulties arose in testing COMT subjects of both the C57BL/6 and 129Sve background on the radial arm maze and object recognition task, some overall observations about the effect of the COMT deletion and its interaction with background can be inferred. Specifically, no differences on the radial arm maze were found between COMT +/+ and COMT -/- subjects maintained on the

C57BL/6 background while genotype differences in subjects maintained on the 129Sve background did exist in the number correct in the first eight choices on testing trials of the radial arm maze. The COMT129 +/+ (129Sve background) had more correct choices in the first eight than the COMT129 -/-. Delay trials of the radial arm maze were not possible with the COMT subjects maintained on the 129 background and object recognition trials were not analyzable in the COMT subjects maintained on the C57BL/6 background making comparison of these tests difficult.

Differences in the effect of COMT deletion in these two studies is most likely caused by differences in background strain. Our previous results indicate that C57Bl/6 subjects have enhanced performance on the radial arm maze compared to 129Sve subjects (see Chapter 2), however, others report that 129sv subjects are enhanced on the spatial radial arm maze (Nguyen et al., 2000). Differences in findings may be due to differences in ambulation, slight differences in task, and differences in substrains of 129 subjects themselves. There are currently at least 14 substrains of 129 (Simpson et al., 1997) and that number may be artificially low since it does not take into account substrains that have been maintained and inbred in individual laboratories. In addition, the 129 subjects in the second study were much less active on the open field than subjects previously studied in our lab and this lack of ambulation was obvious on the radial arm maze as most subjects were unable to complete the task within twenty minutes for any given trial, a highly unusual and slow performance which may reduce the validity of the findings.

Taken together, these results suggest that phenotypic expression of COMT is affected by an interaction with the genetic background strain. Thus phenotypic alterations of null mutants must be carefully examined for possible influences of the background strain on which the mutation is maintained. In conclusion, the expressed COMT -/- phenotype is probably not due to the manipulation of that single gene but to an interaction with the whole genetic background and the environmental conditions to which the organism is exposed. Therefore, genetic background on which null mutants are created and maintained as well as possible environmental influences on phenotypic expression of that null mutation should be carefully considered.

Chapter 5
General Discussion

I. Overview

Up to this point, the influence of environmental conditions on phenotypic expression of specific genetic conditions has been difficult to explore systematically. Several theorists have hypothesized that genetic vulnerability to disease exists, but without that ability to randomly assign subjects of a suspected vulnerable genetic makeup to different environmental conditions it is difficult to assess the exact role of the environment in disease onset. The use of null mutation technology in this collection of studies has begun to provide insight into gene-environment interactions. These studies present evidence that environmental stress has a significant effect on learning and memory which is modulated by genetic background strain and the presence of specific proteins. This work has investigated the influence of background strain (C57BL/6 and 129Sve), a protein acting primarily in the hippocampus- calbindin-D28k, and an enzyme acting primarily in the frontal cortex- catechol-*o*-methyltransferase, on learning and memory and the stress response.

Across the experiments, 14-days of daily restraint stress generally enhanced both object recognition performance and radial arm maze and had minor if any effects on open field activity or neurotransmitter levels. Notably, significant differences in baseline behavioral performance and neurotransmitter levels between background strains were observed. In addition, this collection of studies has found that calbindin-D_{28k} is an important protein in mediating stress

effects on learning and memory and that lack of calbindin-D_{28k} diminished the ability of the system to counteract harmful effects of stress. Alternately, results from these studies indicate that COMT does not plays a significant role in mediating stress effects on learning or memory. What was revealed in the COMT studies, however, was the importance of background strain in expression of the null mutation.

It is clear that genotype can strongly influence behavioral and neurochemical reactivity to environmental effects. The first study compared the effects of stress in two strains of mice commonly used for the creation of transgenic and knockout models, the C57Bl/6 and 129Sve strains. Results of this study indicate that while both strains have similar reactions to stress and show enhanced memory performance, baseline differences in the two strains are significant and should be considered when evaluating behavior. While stress may have similar effects on both strains, evidence from experiments with COMT knockouts indicates that the interaction of these background strains with the homozygous deletion of a particular gene product can have an impact on behavioral assessment. And finally, the calbindin-D28k knockouts provide evidence that deletion of a particular gene product alone may enhance memory performance, but when this deletion is combined with environmental conditions, the effect of the deletion becomes one of impairment in memory performance. Additionally, differences in genetic makeup can affect not only behavioral reactivity but also ability to counteract, 'deal' with, or compensate for these changes in the environment and in homeostasis.

A. Stress effects in controls

Broad examination of control (non-mutant) subjects in this collection of experiments yielded consistent findings regarding stress effects on learning, memory, locomotion, and neurochemistry and support results reported by others. Specifically, in all experiments, stressed subjects failed to gain weight across the stress period. This is consistent with previous findings in rats (Watanabe et al., 1992) and indicates suppression of parasympathetic activation through stress induced arousal of the HPA axis (McEwen et al., 1993). Comparison of stress effects on wild-type subjects show that mice seem to be more sensitive than rats on this parameter (e.g Watanabe et al., 1992). In addition, stress enhanced object recognition performance (specifically, with a one-hour inter-trial delay) in C57BL/6J, 129/SvEvTac, and cal +/+ subjects. Unfortunately, object recognition data was not available from stressed COMT +/+. These are the first studies to investigate the effect of 14-days of restraint stress on object recognition performance. On the radial arm maze comparison of wild-type performance indicates that stress enhanced performance (decreased errors) in the C57BL/6J, 129/SvEvTac and COMT +/+ subjects. Results from the radial arm maze corroborate data from Luine et al. (1994) in which 14-days of restraint stress enhanced radial arm maze performance in rats. Stress did not induce detectable neurotransmitter changes in brain regions examined in the C57BL/6J, 129/SvEvTac, or the COMT subjects. Wild-type calbindin-D_{28k} had somewhat different stress reactivity profiles from wild-type subjects in the other experiments. Specifically, stress increased rather than decreased errors on the

radial arm maze. This was associated with increased rather than no change in norepinephrine levels in the hippocampus and frontal cortex of stressed cal +/+ subjects. While the strain difference and COMT studies failed to find stress induced changes in neurotransmitter levels in several brain regions, significant increases in NE and differences in stress-related radial arm maze performance of stressed cal +/+ subjects suggests small differences between the wild-types used for each study and illustrate the possibility of connection between hippocampal and frontal cortex neurotransmitter levels and spatial memory. Although most of the stress effects are consistent across behavioral and neurochemical data, differences between wild-types across these studies explain small differences from one wild-type to another. Previous studies have found extreme sensitivity to genetic drift caused by inbreeding in different laboratories or breeding facilities **REF. In addition to learning and memory performance, the open field revealed consistent stress effects. In fact, no stress related changes were observed in C57BL/6J, cal +/+, or COMT +/+ subjects. The stress related increase in activity of the 129/SvEvTac found here suggests that the C57BL/6J strain and the wild-type strains on which the null mutations were maintained showed a ceiling effect and therefore no further increases in activity were possible or detectable.

Very few stress related changes were found in neurochemical levels throughout the brain. As discussed previously, this probably indicates the transient nature of the neurochemical changes. Specific differences in neurotransmitter levels in each study between strains or between wild-type and mutants did however yield interesting patterns. Most striking were the large

differences between C57BL/6J and 129/SvEvTac strains in several neurotransmitters including norepinephrine, serotonin, and serotonin turnover (5HIAA/ 5-HT) in most brain regions investigated including the frontal cortex, locus coreleus, and amygdala. Similarly, genotype differences due to the calbindin-D_{28k} null mutation were found in NE in several brain regions including the amygdala, frontal cortex, and locus coreleus in addition to differences in 5HIAA and MHPG in several of these regions. Surprisingly, no alterations in neurotransmitters measured were observed in COMT null mutants.

Overall, examination of the neurotransmitter results suggests significant alterations in noradrenergic and possibly serotonergic pathways which may influence behavioral differences. Remarkably, no differences in dopaminergic levels were observed.

II. Genetic Background Influence on Behavioral and Neurochemical Phenotype

A. Behavior Phenotype

Genetic background strain can influence reactivity to environmental conditions and phenotypic expression of genetic mutations. Results presented provide evidence for differences in behavioral and neurochemical phenotype between the parental strains commonly used to create transgenic and null mutant mice, small differences in reaction to 14-days of chronic stress between these two strains, and differences in expression of the COMT deletion based on the background strain on which the mutation was maintained.

Overall, strain differences in reactivity to different drugs and environmental conditions exist and can have a profound impact on the behaviors of interest (e.g. Crabbe et al., 1999; Crawley et al., 1997). For instance, several studies have characterized strain differences in memory performance and found differences not only among strains but among memory tasks themselves (e.g. Owen et al., 1997). Results presented in the first study (strain differences study) indicate that there are baseline differences in proficiency on the radial arm maze and object recognition task. However, both strains perform the same in response to stress on these learning and memory tasks. Specifically, this study has shown that both the C57BL/6 and 129Sve strains have enhanced performance in response to chronic stress on the spatial radial arm maze and non-spatial object recognition tasks. However, on open field measures of activity, stress affected C57BL/6 and 129Sve strains differently. Specifically, stress increased sector crossings in 129Sve subjects but did not affect C57BL/6. As discussed previously, this difference probably reflects a ceiling effect in activity in the C57BL/6 strain. In addition to behavioral effects, no detectable stress related changes were found in neurochemical levels in brain regions investigated.

It is important to note that, although strains reacted similarly to stress on measures of the radial arm maze, object recognition task, and neurochemical levels, significant differences in baseline behavioral and neurotransmitter levels exist between the C57BL/6 and 129/SvEvTac strains. Our results reveal that C57BL/6 subjects made fewer total errors than 129/SvEvTac subjects on the radial arm maze, confirming previous results in which C57BL/6 perform better

than 129Sve on a different spatial memory task, the Barnes maze (Nguyen et al., 2000). In contrast, C57BL/6 subjects spent less time exploring the novel object in the object recognition task, revealing the dichotomy in the types of memory the radial arm maze and object recognition task are assessing.

In contrast to the radial arm maze, 129Sve perform very well on the object recognition task. Although initially all subjects discriminated between the old and new objects on the object recognition task with an inter-trial delay of 10 minutes, extension of the inter-trial delay period to 1-hour on a subsequent trial revealed strain differences. The 129/SvEvTac subjects spent more time exploring the novel object at this one-hour delay compared to C57BL/6J subjects, despite the 129/SvEvTac subjects spending less time exploring the objects on the field during the sample trial (T1). This difference in initial exploration time is consistent with higher anxiety level observed on the open field and was controlled for statistically in the analysis of discrimination between the old and new objects. These results suggest better memory performance on this non-spatial task by the 129/SvEvTac subjects compared to the C57BL/6J subjects.

Differences between the non-spatial object recognition and spatial radial arm maze suggest reasons for divergent performance differences between the two strains. Not only does the object recognition task relies less on ambulation than the radial arm maze and not require food restriction, but the object recognition task is considered a non-spatial memory task which is disrupted by destruction of the entorhinal cortex (Ennaceur et al., 1996; 1997). In contrast, performance on the radial arm maze requires spatial memory and hippocampal function (Olton et

al., 1979). The diverse loci of these types of memory may illuminate strain differences in brain morphology similar to differences already observed in corpus callosum structure between these strains (Balogh et al., 1999). These results confirm the need for several tests of memory to accurately memory performance in the two strains or in a mixed background.

Regardless of memory task, stress enhanced both the C57BL/6J and 129/SvEvTac strains. On the object recognition task with a one-hour inter-trial delay, stressed subjects of both strains spent significantly more time exploring the novel object than non-stressed subjects. During delay trials of the radial arm maze, stressed subjects made significantly fewer errors overall than non-stressed subjects. Since stress effects were consistent in these measures, 14 days of chronic stress may be a useful tool for assessing the potential effect of environmental factors in these strains as well as in null mutant and knockout models created on these backgrounds.

Overall, differences in activity on the open field indicate that C57BL/6 are more active and ambulatory than 129Sve. This supports findings by Homanics et al (1999) that 129 strain mice are less active on the open field and may be more anxious. Therefore, assessment of learning and memory in null mutant and transgenic models maintained on mixed backgrounds of these strains may require consideration of these motor differences and potentially may require statistical controls for differences in activity

More striking than behavioral differences among the strains were differences in neurotransmitter levels among several brain regions in C57BL/6J

and 129/SvEvTac subjects. Specifically, levels of norepinephrine ranged from seven to twelve times higher across brain regions in 129/SvEvTac compared to C57BL/6J subjects. In addition, significant differences in serotonin in many of the same regions were observed with 129/SvEvTac subjects generally having much higher levels than C57BL/6J subjects. As with behavioral data, differences in stress reactivity in the two strains was not different in most neurochemical measures. These findings have previously been discussed in relation to behavioral outcomes and many of the differences in neurotransmitter levels reflect previously found differences in behavior. However, the large variations in neurochemical levels between the strains suggest extreme differences which may exist in neuroanatomical structures. For instance, recent studies have shown significant reductions in corpus callosal density in most strains of 129 mice subjects compared to C57BL strains (Balogh et al., 1999). Though the experimenter observed no gross differences during tissue dissection or punching, future studies of neuroanatomical differences, particularly of regions subserving areas of the brain involved in memory, may reveal significant differences between the two strains.

B. Background Genotype Interactions with COMT Expression

The phenotypic expression of a null mutation can depend on the interaction of that mutation with the genetic background on which it is maintained. Results from studies in COMT knockouts indicate that the effect of COMT deletion in females depends on the background strain on which the knockout is maintained. Specifically, significant differences between COMT +/+

and COMT -/- subjects maintained on the C57BL/6 background exist were different from those observed between COMT +/+ and COMT -/- subjects maintained on the 129Sve background. The most striking difference in phenotypic expression of the COMT -/- genotype was the inability to accurately assess performance on the spatial radial arm maze (in COMT +/+ and COMT -/- subjects maintained on the 129Sve background) or the non-spatial object recognition task (in the COMT +/+ and -/- subjects maintained on the C57BL/6 background). Although difficulties arose in testing COMT subjects of these backgrounds on the radial arm maze and object recognition task, some overall observations about the effect of the COMT deletion and its interaction with background can be inferred. Specifically, no differences on the radial arm maze were found between COMT +/+ and COMT -/- subjects maintained on the C57BL/6 background while genotype differences in subjects maintained on the 129Sve background did exist in the number correct in the first eight choices on testing trials of the radial arm maze. Specifically, the COMT +/+ (129Sve background) had more correct choices in the first eight than the COMT -/- (129Sve background). Delay trials of the radial arm maze were not possible with the COMT subjects maintained on the 129 background and object recognition trials were not analyzable in the COMT subjects maintained on the C57BL/6 background, making comparison of these tests difficult. The inability to accurately assess object recognition performance in COMT +/+ and -/- subjects maintained on the C57BL/6 background suggest either significant anxiety (not detectable on the open field) or an impairment in attention. Strikingly, these COMT (C57BL/6 background) were extremely active

on the open field, particularly when compared to C57BL/6J subjects from the first study. For instance, COMT +/+ and -/- (C57BL/6 background) had almost twice as many sector crossings on the open field as C57BL/6J subjects from the first experiment. Yet, when placed in the field with the objects, the COMT +/+ and -/- (C57BL/6) showed little to no activity. Procedures aimed at reducing possible anxiety in the object recognition task, in addition to exposure to the field prior to testing, included acclimating the subjects in a clear box on the field for 15 seconds prior to the start of the trials. These procedures did not appear to increase exploration as most subjects spent fewer than 7 seconds exploring both objects combined. Observational reports from the experimenter during these object recognition trials reveal that subjects tended to spend the majority, if not all, of the 5-minutes of T1 and T2 in a corner as far away from the objects as possible. This reaction to objects in the field was not observed in COMT +/+ or -/- subjects of the 129Sve background (nor was it observed in any other experiments).

III. Calbindin-D28k

Investigation of the effects of calbindin-D_{28k} deletion reveals that deletion of a gene product, while not deleterious to basic performance, can impair an organism's ability to counteract stress. Results indicate that there is a different optimal range of calbindin-D_{28k} expression in the brain for locomotor activity, learning and memory, as well as resistance to chronic environmental stress. While calbindin-D_{28k} null mutation enhanced object recognition performance and maintained radial arm maze performance, stress impaired cal -/- subjects on both tasks. Thus, calbindin-D_{28k} presence in the central nervous system slightly

decreased memory performance, it may be necessary for its neuroprotective properties related to stress effects. There is also some evidence that expected sex differences in spatial memory performance are altered in cal -/-.

A. Effect of calbindin-D_{28k} null mutation

Cal -/- performed better than cal +/+ controls on the non-spatial object recognition task and performed the same on the spatial radial arm maze. Cal -/- spent significantly more time exploring the novel object than cal +/+ controls during a one-hour inter-trial delay assessment. On the spatial radial arm maze task, no genotype differences were found through acquisition, testing, or delay trials. Further, as expected due to the abundance of calbindin in the cerebellum of normal animals, cal -/- were less active on all measures of the open field.

While the possibility existed that differences in activity may have confound interpretation of behavior performance, our results have found that the cal -/- who had *decreased* activity on the open field had *enhanced* performance on the object recognition task. Further, there was no evidence that a decrease in activity in cal -/- affected exploration of objects in the object recognition task as there was neither a difference in initial exploration time in the sample trial nor a difference in total exploration time during the recognition trial. In addition, potential differences in activity did not affect interpretation of radial arm maze data as the correlation between time to complete the task and performance was very low for both cal +/+ and cal -/- and this possible influence was controlled statistically by incorporating activity as a covariate in the analysis.

In contrast to the object recognition performance and as expected, cal -/- did not perform better than cal +/+ on the radial arm maze. These findings are similar to those of Molinari et al. (Molinari et al., 1996) who reported only a slight impairment in acquisition of the radial arm maze in calbindin-D_{28k} deficient mice. As discussed by Molinari et al. (1996), calbindin-D_{28k} deficient mice did not show general learning differences from controls but had possible selective impairment in acquisition of spatial processing. Impairments in their study on the radial arm maze were found in only 2 out of 10 acquisition trials (Molinari et al., 1996). Minor impairment was also found in a select 2 out of 16 trials of the spatial Morris water maze (Molinari et al., 1996). Our testing extended beyond acquisition, and while our results failed to pick up this slight impairment, further testing revealed no additional differences between cal +/+ and cal -/-. minor differences in procedures between the studies include differences in the amount of calbindin-D_{28K} which was expressed in the calbindin-D_{28k} null mutant mutants. The mice used in the Molinari et al. (1996) study were created with an antisense gene insertion and were not a complete null mutant of the calbindin-D_{28K} protein. In addition, subjects in the Molinari et al. (1996) study were water deprived and reinforced with a sugar solution while in our study the mice were food deprived and reinforced with peanuts. These small differences in calbindin-D_{28k} expression and reinforcement procedures may have resulted in the minute differences in the results of the two studies.

The main genotype difference in this study was found in object recognition performance in which cal +/+ had inferior performance compared to

cal -/-. However, no significant differences were found in radial arm maze performance. These results are consistent with dichotomous performance on spatial versus non-spatial memory tasks previously reported in calbindin-D_{28k} deficient mice (Molinari et al., 1996). Since each of these tasks relies on different brain regions and calbindin-D_{28k} expression varies from one brain region to another, it is not surprising that there are differences in the effect of the deletion on these tasks. Object recognition is a non-spatial, non-reward task that is dependent, in part, on attentional aspects of the frontal cortex (Ennaceur et al., 1994; Ennaceur et al., 1997) and the entorhinal cortex (Ennaceur et al., 1996). Conversely, the radial arm maze requires spatial cues and is dependent on hippocampal learning (see Robbins et al., 1997 for review). Other differences between the tasks include that the radial arm maze requires extensive training, food deprivation, and reward based learning whereas the object recognition task is non-reward based, does not require food or water restriction, and can be assessed without prior training of the animals. In summary, our results indicate that deletion of calbindin-D_{28k} did not enhance hippocampal based learning whereas this deletion did enhance performance in fronal and entorhinal cortex based learning.

B. Stress Effects related to calbindin-D_{28k}

Stressed subjects of both genotypes failed to gain weight across the stress period, thus confirming that the 14-days of restraint was an effective stressor. These results corroborate previous results that stress can lead to weight loss (e.g.

Willner et al., 1996; Magarinos et al., 1995; Krugers et al., 1996; Beck et al., 1999). In learning and memory tasks, stress had different effects on cal -/- and cal +/+ subjects depending on the task itself. Stress impaired memory performance of cal -/- on both the object recognition and radial arm maze tasks. Whereas in cal +/+ subjects, stress enhanced object recognition and impaired radial arm maze performance. These differences in the effects of stress may be due to a number of distinguishing features of each task. Differences in stress effects on task performance suggest that stress enhanced frontal cortex dependent attention and learning but impaired hippocampal learning in cal +/+ subjects. In contrast, stress impaired performance on both the radial arm maze and object recognition tasks in cal -/- mice. Cal -/- subjects may have altered intracellular calcium levels and thus altered release of and sensitivity to excitatory neurotransmitters necessary for learning and memory (Brager et al., 2000). However, when confronted with stress, subjects with null mutation of calbindin-D_{28K} may lack the ability to mediate calcium influx and thus have less resistance to the damaging effects of increased excitatory neurotransmitter release associated with chronic stress. Recent studies investigating the excitatory pathways have found that kainic acid, a glutamate agonist, does not appear to affect vulnerability of the CA1 area of the hippocampus in calbindin-D_{28K} -/- subjects (Gary et al., 2000), but no studies have investigated similar vulnerability in a calbindin-D_{28k} -/- system or vulnerability under stress conditions. As suggested by our data discussed below in the neurochemistry section and previous research (Klapstein et al., 1998), the integrity of intracellular mechanisms including action potential properties may be

altered in cal-/- subjects. These internal mechanisms may not be resistant to stress induced changes, a finding which is supported by our neurochemical results showing differential alterations in neurotransmitter levels and metabolism following stress in cal -/- compared to cal +/+ subjects.

Changes in calcium levels due to calbindin-D_{28k} presence or absence may alter properties of neurons in brain regions responsive to stress and important in learning and memory (Klapstein et al., 1998). Recent evidence shows alterations in calcium levels in neurons of cal -/- subjects (Pasti et al., 1999; Klapstein et al., 1998) which may alter neuronal function and structure. Agonist induced increases in calcium have been shown to inactivate NMDA receptor-channels in hippocampal and cerebellar neurons (Legendre et al., 1993; Medina et al., 1996; Medina et al., 1995; Rosenmund et al., 1995). This down regulation of NMDA receptor activity may partially account for the enhancement in learning of cal -/- since the calbindin-D_{28K} null mutant system may be adapted to respond to circulating levels of glutamate. However, when overloaded by chronic stress, down regulation of these receptors may not be sufficient to counteract the overstimulating effects of stress. Recent evidence from calbindin-D_{28K} deficient mice indicates that there is in fact a decrease in NMDA receptor mediated responses while non-NMDA responses are increased (Jouvenceau et al., 1999). This decrease could not be ascribed to changes in NMDA channel properties of the calbindin-D_{28K} null mutant subjects (Klapstein et al., 1998). Neurons from calbindin-D_{28K} null mutants (cal -/-) have been shown to have increased depolarization induced calcium rises and prolonged elevation of the calcium

signal (Airaksinen et al., 1997; Pasti et al., 1999). Thus, changes in glutamatergic neurotransmission may be altered in calbindin-D_{28K} null mutants (cal -/-) and may be caused by lack of calbindin-D_{28K} in the cell itself. Altered glutamate transmission may increase vulnerability of the cell to stress. Alternatively, calbindin-D_{28K} null mutants (cal -/-) may upregulate other calcium binding proteins. Although no upregulation of calretin, parvalbumin, calbindin-D_{9K}, calmodulin, or S100β was observed compared to cal +/+ subjects, the possibility that another unexamined or unknown calcium binding protein is upregulated cannot be dismissed (Airaksinen et al., 1997).

C. Sex Differences

Consistent with previous reports, males were less active on the open field and had fewer errors on the radial arm maze compared to females (Luine et al., 1994b; Beck et al., 1998; Williams et al., 1990). The sex differences on the radial arm maze were somewhat inconsistent in cal +/+ and cal -/-. Specifically, there was no significant sex difference in performance on acquisition trials of the radial arm maze in cal -/- subjects, suggesting that calbindin-D_{28K} may be affecting sexual differentiation of brain regions involved in spatial memory acquisition. This is consistent with recent findings that males and females express different levels of calbindin-D_{28k} in several brain regions (Brager et al., 2000; Sickel et al., 2000; Brager et al., 2000; Taylor et al., 1999; Watson et al., 1998; Lephart et al., 1997; Lephart, 1996). Lack of calbindin during development may therefore have slightly attenuated sexual differentiation of brain regions. Further studies on the

specific effects of calbindin-D_{28k} deletion on sexual differentiation may elucidate the basis for the decrease in sexual dimorphism of cal -/-.

As discussed above, changes in glutamatergic signaling caused by calbindin-D_{28k} absence may be affected by and may affect neurochemical signaling (Jouvenceau et al., 1999). Differences in behavior may be partially attributed to observed changes in monoamine and metabolites in several brain regions in cal -/- compared to cal +/+. As predicted and consistent with behavioral differences, significant differences between cal +/+ and cal -/- subjects emerged for several monoamines and their metabolites in several brain regions. Most notably, norepinephrine (NE) was lower in cal -/- subjects in three brain regions investigated: the frontal cortex, amygdala, and locus coreleus. In addition, in the amygdala, levels of the NE metabolite, MHPG, were lower in cal -/- subjects. Previous studies have investigated the potential relationship between NE and learning and found similar results. Specifically, Landers and Sullivan (1999) showed that injections of NE could block acquisition of conditioned responses. Similarly, in our study, the cal +/+ subjects, which exhibited higher levels of NE, showed worse performance on the object recognition task compared to cal -/- subjects. The object recognition task has previously been shown to rely, in part, on frontal cortex function (Ennaceur et al., 1997). Thus differences in NE in the locus coreleus and the frontal cortex upon which it projects may affect attentional components necessary for memory on a non-spatial task.

Notably, reactivity of monoamine systems to stress in cal -/- subjects was *not* similar to reactivity in cal +/+ subjects. Specifically, in the hippocampus,

several monoamines were elevated in stressed cal +/+ subjects but did not show similar increased in stressed cal -/- subjects. Specifically, levels of NE and its metabolite, MHPG, were higher in stressed cal +/+ subjects than any other group. Results from these cal +/+ subjects confirms previous findings that a variety of chronic stressors increase NE release (for review see Shanks et al., 1991; Shanks et al., 1994; Beck et al., 1998; Weiss et al., 1988). As suggested by Anisman and Zacharo (1997), the increase in NE in response to chronic stress enables the animal to deal with environmental demands. Our results suggest that the increase in NE in response to chronic stress permitted the cal +/+ subjects to perform effectively on the non-spatial object recognition task. However, stressed cal -/- subjects did not exhibit increased NE in response to chronic restraint and displayed poor performance on both memory tasks. This inability to increase NE may have led to diminished ability to cope with the environmental challenges and memory demands of both the object recognition and radial arm maze tasks. Further investigation into the mechanism by which stress increases NE release and its specific role in memory function may provide an understanding of the role of calbindin-D_{28K} or calcium mobilization in this response.

One of the cellular mechanisms that may influence neurotransmitter levels is calcium mobilization. Previous studies have found that decreased calcium mobilization impairs avoidance performance and spatial radial arm maze performance (Ohnuki et al., 1996). It is therefore possible that any changes in calcium mobilization in cal -/- may be influence memory processes (Jouvenceau et al., 1999; Klapstein et al., 1998). Further, recent studies with cal -/- have shown

alterations in action potential firing (Klapstein et al., 1998) and long-term potentiation, a mechanism considered important in memory formation (Molinari et al., 1996). Our results indicate that there are significant differences in learning and memory processing and in the stress effects on learning in cal -/- subjects which may be influenced by changes in calcium mobilization, action potential firing, as well as long term potentiation.

Several studies have reported highly transient changes in monoamine concentrations depending on time post-stress when the monoamines were measured (Beck et al., 1998). Dopamine (DA) and serotonin (5-HT) appear be more susceptible to transient changes (Finlay et al., 1995). Therefore, it is not surprising that few changes in either DA or 5-HT or their metabolites were detected in this study since brains were extracted almost 2 weeks after the application of stress. However, our results do not preclude changes in these monoamines at earlier intervals following stress or in other brain areas.

In conclusion, our data suggest that calbindin-D_{28k} is involved in the stress response, several pathways underlying this response, as well as behavioral functioning. Therefore, an optimal amount of calbindin-D_{28k} may be necessary for different types of learning, memory, locomotion, and response to stress. This optimal level may not be the same for all of these processes. Calbindin-D_{28k} deletion enhanced performance on the non-spatial memory task and did not affect measure of spatial memory performance compared to wild-type controls. However, chronic stress appears to compromise the ability of the calbindin-D_{28k} null mutant system to compensate as mice lacking calbindin-D_{28k} had impaired

performance on both memory tasks following stress and lacked neurochemical compensation to the environmental stress. Inability to compensate for stress in calbindin-D_{28k} -/- subjects may be due to differences in intracellular calcium signaling, neurotransmitter release and use, and action potential characteristics. Our neurochemical evidence suggests significant disruption of the noradrenergic system which may underlie differences in stress reactivity and learning and memory between cal +/+ and cal -/-. Further in vivo and in vitro studies are clearly necessary to understand these results and clarify which neural mechanisms underlie genetic and environmental effects. This study illustrates the usefulness of studying the stress response in null mutant models. Taken together, these results suggest that alteration of a system involved in central nervous system processes may not immediately result in impairments but may compromise the ability of the organism to deal effectively with environmental demands.

IV. COMT

Work with the COMT knockout has provided a comparison of gene deletion effects on two common genetic backgrounds. The first study provided information about the role of COMT in the stress response. These COMT null mutants (C57BL/6 background) had less locomotor activity on the open field, whereas stress returned these knockouts to normal levels of ambulation. There were no significant differences between COMT +/+ and -/- groups in testing or delay trials of the spatial radial arm maze task. Data indicate that COMT deficiency does not severely impair subjects in spatial memory and stress may be interacting with the COMT deletion to enhance performance slightly. Stress

subjects of both genotypes (COMT +/+ and -/-) had improved performance (fewer errors) on radial arm maze delay trials. Data from control subjects (COMT +/+) on the radial arm maze testing trials and delay trials verifies previous findings that fourteen-day restraint stress enhanced memory performance (Kneavel et al., submitted manuscript; Luine et al., 1994). Not only did COMT deficiency not severely impair subjects in this spatial memory task, stress enhanced performance. Further testing at longer delays may clarify the role of this enzyme in the stress response cascade since longer delays may push the limits of the memory system in all subjects. Additionally, lack of differences in neurotransmitter levels in the frontal cortex, hippocampus, or amygdala corresponds to lack of profound changes in behavior suggesting that even with the additional insult of environmental stress, COMT -/- females maintain homeostasis and thus do not show changes in catecholamine levels or turnover. This is in contrast to male COMT -/- subjects which, in previous studies exhibited significant increases in dopamine levels in the frontal cortex and changes in HVA/DOPAC ratios (an indication of decreased turnover and catabolism by COMT) (Gogos et al., 1998).

Previously reported (Gogos et al., 1998), sexually dimorphisms in COMT knockouts may be due to possible differences in hormonal environments. This study provides further evidence that female hormones may further protect COMT -/- from not only deletion of COMT but also environmental stressor effects, particularly in memory processing. COMT -/- subjects were not significantly impaired on the spatial radial arm maze and stress did not further impair these COMT -/- subjects in memory performance, in fact, stress enhanced performance

on the radial arm maze. However, a significant interaction between stress and genotype on the open field confirms that stress does have a somewhat different effect in COMT -/- subjects that was detected by this measure of activity. The differences in activity between COMT -/- and COMT +/+ females may be due to previously reported increased anxiety behavior and subsequent decreased ambulation in the light portion of the light/ dark shuttlebox (Gogos et al., 1998). To decrease anxiety, all subjects in this experiment were handled daily before behavior testing began and were tested under dim red light conditions. Despite these efforts to control anxiety, results from the open field, mimic results found previously by Gogos et al. (1998) that decreased activity in female COMT -/- subjects was directly related to increased anxiety. These results suggest that the open field procedure utilized in this experiment was anxiety producing to the COMT -/- subjects. Stress attenuated this anxiety related decrease in behavior in the COMT -/- subjects. This may reflect a general increase in activity level commonly observed after stress (Beck et al., 1998). Further, testing on the radial arm maze did not reflect this anxiety influence since subjects had multiple exposure to the maze during training before testing began which has been suggested to effectively reduce activity related differences (Homanics et al., 1999).

Results of COMT experiment indicate that this enzyme does have an effect on learning and memory and there is an interaction with stress. Proposed work would include a more comprehensive assessment of the influence of the COMT enzyme by including male subjects. Previous work with males and

females has shown significant differences on several behavioral tasks and there may be similar sex differences in learning and memory as well as the stress response. This would provide further insight into the mechanism by which COMT functions in the system. If females are less affected by the null mutation than males consistent with neurotransmitter differences, a compensation mechanism may be acting in females which could be useful for combating the effects of COMT deficiency. In addition, future studies on the direct effect of stress on neurochemical levels in these knockouts through in vitro dialysis or measurement of levels directly after stress (without intervening behavioral testing) may provide further insight into the mechanisms of COMT, dopamine, norephinephrine, epinephrine, metabolites, and frontal cortex functioning in the stress response.

Results from both studies indicate that effect of COMT deletion in females depends on the background strain on which the knockout is maintained. Specifically, significant differences between COMT +/+ and COMT -/- subjects maintained on the C57BL/6 background exist but these differences were not the same as those found in COMT +/+ and COMT -/- subjects maintained on the 129Sve background. One of the most obvious differences between subjects of both studies is the lack of data from one of the memory tasks in each of the studies. Specifically, no data is available from the object recognition task in COMT +/+ or -/- subjects maintained on the C57BL/6 background possibly for reasons associated with anxiety that were discussed previously. And in the COMT +/+ and -/- subjects maintained on the 129Sve background, data from the radial arm maze was difficult to attain and delay trials were impractical. However, with

these constraints in testing, some overall observations about the effect of the COMT deletion and its interaction with background can be inferred. Specifically, no differences on the radial arm maze were found between COMT +/+ and COMT -/- subjects maintained on the C57BL/6 background while genotype differences in subjects maintained on the 129Sve background did exist in the number correct in the first eight choices on testing trials of the radial arm maze. The COMT +/+ (129Sve background) had more correct choices in the first eight than the COMT -/-.

Experimental results presented here exemplify the complex problems associated with modeling how environmental stimuli or conditions can influence behavior.

V. Summary

The experiments discussed here exemplify some of the limitations and caveats of assessing the effects of a null mutation. These studies illustrate the complex nature of phenotypic expression of a particular gene product. Not only is the phenotypic expression influenced by the genetic background used to create the null mutation (which is often more than one strain) but it is also influenced by the environmental conditions to which the null mutant is exposed. Care should thus be taken when arriving at conclusions about the effect of the null mutation and inferring from these results the function of this gene product in behavioral, endocrine, and neurochemical systems. That said, however, null mutation is a useful new tool when used in combination with existing methods including pharmachology, lesion studies, labelling studies, neuroimaging, and electrical

recording among others for determing function. This null mutation technology can start to provide a link between gene and environment interactions.

One of the clearest difficulties in these studies was the establishment of these behavioral paradigms. With the influx of null mutants available, the need for behavioral tests for these mice has increased. It is not always sufficient to simply scale down apparati used for rats. Often there are unknown factors which must be worked out before a paradigm will be suitable for testing mice. Whereas the radial arm maze had been well established in mice e.g. (Ohnuki et al., 1996), (Whishaw & Tomie, 1996) (Roullet & Lassalle, 1992) (Roullet, Lassalle, & Jegat, 1993) less information was available on procedures for testing non-spatial memory utilizing the object recognition task. Further, the few studies that had used this technique in mice had all used very different procedures e.g. (Messier, 1997; Dodart, Mathis, & Ungerer, 1997). As discussed in reference to the COMT experiment, difficulty in measuring object recognition memory may be due to anxiety produced by the objects themselves. Specifically, in the COMT study, subjects were extremely active on the open field, yet when placed in the same field with two objects, subjects appeared 'frozen' and remained in a corner of the field as far away from the objects as possible. While considerable testing took place prior to these experiment to establish pairs of objects that were both interesting and equally attractive to mice, the results from the COMT experiment suggested that additional modification was needed. This 'anxiety-like' response to the objects may have been a result of the specific objects used or a reaction found specifically in these subjects.

In addition to establishing working protocols for these behavioral tests, significant strain differences exist which can require careful examination of the measurements used in order to detect large differences that often exist between strains. For instance, in these experiments, the 129Sve strain in the first study is much less active on the open field and stress increased activity in this strain. Similar increases in activity were not observed for any other stress group in any of the other experiments suggesting that non-stressed control groups may have exhibited maximum activity and any stress effects on activity would not be detectable. Thus, interpretation of behavioral 'effects' need to take into consideration some of the aspects of the task itself.

Further, as illustrated in these studies, large behavioral and neurochemical differences exist between mice strains. If different strains are used to extract the ES cells and to maintain the null mutation, confounds can arise in interpretation of the null mutation behavior as directly due to the mutation and not an interaction of the mutation with the rest of the genotype (Crawley, 2000). Specifically, the COMT null mutant maintained on the C57BL/6 background exhibited a different phenotype than the COMT null mutant maintained on the 129Sve background.

VI. Future Directions

With these limitations in mind, however, there are several follow up studies which could clarify the effect of stress on learning and memory in mice. First, further studies of the 14-day restraint stress in other mouse strains will clarify the effect of stress even under diverse genetic conditions. In addition, further understanding of the stress reaction in female mice is needed. Recent

evidence has illustrated sex differences in stress reactivity in rats which may be related to hormone environment (Bowman, Zrull, et al. 2001), no studies are currently available on the effects of stress in female mice. Lastly, no studies on the effects of longer stress periods (i.e., 21 days) on learning and memory performance are available in mice, however, these effects are well known in rats (Beck et al., 1998; Luine et al., 1994a).

In addition to studies of the stress response in wild-type mice, several follow up studies in the null mutants could increase understanding of these proteins. Future studies involving calbindin-D_{28k} null mutants could provide more insight into the mechanisms of this protein. Specifically, further investigation of potential sex differences in behavior, neuroanatomy, and neurochemistry in the calbindin-D_{28k} null mutants may increase understanding of the potential role of this protein in sexual differentiation of the central nervous system. In addition, studies of the effects of shorter stress periods, kainic acid exposure, and glucocorticoid injection or ingestion in these calbindin-D_{28k} null mutants may clarify not only the role of this protein in the stress response, but also its neuroprotective properties. Lastly, future studies of the stress effect in male COMT null mutants in addition to the current studies of females may further increase understanding of this mechanism in both sexes.

While more experiments will increase understanding of the mechanisms discussed, this collection of studies has accomplished the aims defined initially. Specifically, general understanding of the stress effect on learning and memory in two strains commonly used to create null mutants was attained. Behavioral and

neurochemical protocols were established for testing stress effects on neurocognitive components in mice and particularly in null mutants. Further, The effect of chronic stress in null mutants was established as a significant factor in learning, memory, locomotion, and neurochemistry. And finally, the relative importance of both calbindin-D_{28k} and COMT in central nervous system functioning, stress reactivity, and behaviors were established.

VII. Conclusion

These results answered a number of the questions originally posed in this thesis. First, specific differences were found between the two strains commonly used in the creation of null mutant and transgenic models, the C57BL/6 and 129Sve strains. However, no specific differences in learning and memory performance, locomotion, weight, or neurochemical changes were found between these strains in response to stress. With regard to the calbindin-D_{28k} null mutants, the effect of the mutation on learning and memory revealed that deletion of this protein enhanced non-spatial memory performance, while no differences in spatial memory performance were observed compared to wild-type controls. Further, in the calbindin-D_{28k} null mutants, stress impaired both spatial and non-spatial memory performance and was not associated with increases in norepinephrine. These results suggest that calbindin-D_{28k} null mutation affected memory performance and stress vulnerability which may have been related to alterations in neurotransmission particularly of the noradrenergic system and were not likely due to influences of the background strains. In the final study, the influence of the background strain on expression of null mutation of COMT revealed that

differences between the null mutants and wild-types were observed when the mutation was maintained on the 129Sve background. Specifically, the COMT null mutants showed slight impairments on the non-spatial memory task. However, analysis of any stress effects in these COMT null mutants revealed no specific effects of stress on learning and memory, neurochemistry, or other measures.

In conclusion, overall results have found that significant differences exist between common progenitors of null mutants, the C57BL/6 and 129Sve strains, in learning, memory, and locomotor behavior as well as neurochemical profile; however, stress responses on these measures are essentially the same. With these behavioral and neurochemical profiles understood, it was then possible to proceed with characterizing the specific effects of null mutation of both calbindin-D_{28k} and COMT, proteins hypothesized to be involved in the stress process. The second study then established calbindin-D_{28k} as an important protein in learning and memory and in protection against stress related impairments. Specifically, calbindin-D_{28K} null mutants exhibited enhanced memory performance compared to wild-type controls, however, stress impaired performance in these null mutants. These alterations in performance were associated with specific neurochemical changes, particularly in noradrenergic systems. Finally, while no specific effects of COMT null mutation on learning and memory were observed, differences in phenotypic expression based on genetic background on which the mutation was carried provide insight into gene-mutation interactions, which can influence interpretation of null mutation effects.

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